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(51) International Patent Classification ^{6} : A61K 31/70	**A1**	(11) International Publication Number: **WO 98/50047** (43) International Publicati n Date: 12 November 1998 (12.11.98)

(21) International Application Number: PCT/US98/09031

(22) International Filing Date: 8 May 1998 (08.05.98)

(30) Priority Data:
60/046,030   9 May 1997 (09.05.97)   US
60/061,716   10 October 1997 (10.10.97)   US

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS AND COMPOSITIONS FOR REDUCING ISCHEMIC INJURY OF THE HEART BY ADMINISTERING ADENOSINE RECEPTOR AGONISTS AND ANTAGONISTS

(57) Abstract

Compositions and methods for reducing or preventing ischemic damage of the heart are disclosed. A preferred embodiment of the invention comprises the simultaneous administration of specific A3/A1 receptor agonists, to patients suffering from ischemic damage or at risk for the same. In yet another embodiment of the invention, a binary conjugate which acts as an agonist for the A3 receptor and an antagonist at the A2a receptor, is administered to reduce or prevent ischemic damage to the heart.

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METHODS AND COMPOSITIONS FOR REDUCING
ISCHEMIC INJURY OF THE HEART BY ADMINISTERING ADENOSINE RECEPTOR AGONISTS AND ANTAGONISTS

FIELD OF THE INVENTION

The present invention relates to therapeutic methods for protecting the heart from ischemic injury.

10   More specifically, the methods of the invention involve administering agonists and antagonists or binary conjugates thereof which selectively activate or inhibit adenosine receptors simultaneously thereby enhancing the protective effects of preconditioning and rendering the

15   myocardium more resistant to ischemia.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application by numerals in parenthesis in order to more

20   fully describe the state of the art to which this invention pertains. Full citations for these references are found at the end of the specification. The disclosure of each of these publications is incorporated by reference herein.

25   Adenosine is released in large amounts during myocardial ischemia and can mediate potentially important protective functions in the cardiovascular system (1,4,5,7,9,14, 17,18,19,25). Previous studies have shown that adenosine receptor agonists can

30   precondition the heart when given before the onset of ischemia (4,5,9,14,17,18) and can cause reduction in

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infarct size or improvement in left ventricular function
when given during reperfusion (1,19) or during both
low-flow ischemia and reperfusion in isolated perfused
heart (6,21,22). While activation of adenosine A1 and

5   A3 receptors has been shown to mimic the cardio-
protective effect of preconditioning (3,10,23,24), their
roles in mediating the protective effect of adenosine
administered during ischemia have not yet been fully
elucidated. Further, the cardioprotective effect of

10   exogenous adenosine infused during ischemia in the
intact heart may be exerted at the level of coronary
vasculature, circulating neutrophils, or cardiac
myocytes.

     Our previous studies have characterized a cardiac

15   myocyte model of injury, which is induced by exposure of
myocytes to prolonged hypoxia in glucose-free media
(16,23). Use of this model has facilitated the
identification of compounds that enhance the protective
effects of preconditioning and also increase myocardial

20   resistance to ischemia.

SUMMARY OF THE INVENTION

     The present invention provides methods for
preventing or reducing ischemic damage of the heart. In

25   conducting research leading up to this invention, it was
discovered that simultaneous activation of A3 and A1
receptors enhances the protective effects of
preconditioning and increases myocardial resistance to
ischemia. The concept underlying the present invention

30   is the use of specific agonists which simultaneously
activate these two adenosine receptors. Concomitant
activation of the two receptors is believed to produce a
synergistic effect enhancing the cardioprotective
effects of preconditioning and increasing myocardial

35   resistance to ischemia.

     According to a preferred embodiment, the invention
involves administration of specific A1/A3 agonists, such

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as N^6-(2-trifluoromethyl)(carbamoyl)adenosine-
5'uronamide or N^6-(3-iodophenyl)(carbamoyl)
adenosine-5'uronamide during ischemic attacks, or at
risk for ischemic damage. The agonists of the invention

5   may be delivered prior to a surgical procedure. They
may also be administered to a patient to prevent or
reduce the severity of ischemic damage during surgery.
Additionally, the A3/A1 agonists may be administered
following surgical procedures to reduce the risk of

10   post-surgical ischemic complications. Finally, the A3/A1
agonists may be administered to patients with angina or
to patients during a myocardial infarction. The angina
may be chronic and stable, unstable, or post-myocardial
infarction angina.

15   In yet another embodiment of the invention, a
series of water-soluble MRS compounds are contemplated
to be within the scope of the present invention. These
compounds selectively activate the A3 receptor. Because
the compounds of the invention do not cross the

20   blood-brain barrier, the deleterious effects associated
with A3 receptor activation in the brain are avoided.
The MRS compounds will be used in conjunction with the
A1 agonists of the invention to prevent or reduce
ischemic damage to the heart.

25   Another preferred embodiment of the invention
comprises novel binary conjugates which bind two
adenosine receptors simultaneously. Exemplary binary
conjugates of the invention contain moieties that act as
agonists at both of the A1 and A3 adenosine receptors,

30   such as MRS 1543. A second exemplary conjugate, MRS
1528, acts simultaneously as an agonist at the A3
receptor and as an antagonist at the A2a receptor.
Methods are disclosed herein for the administration of
these binary conjugates to protect the heart against

35   ischemic damage.
         Methods of simultaneous administration of the A3
and A1 agonists or the binary A3 agonist/A2a antagonist

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or the binary A3 agonist/A1 agonist of the invention
include direct perfusion of the organ during surgery and
intravenous administration. Additionally, the agonists
and antagonists of the invention may be administered to
5   patients in tablet form in an amount effective to
prevent or reduce ischemic damage to the heart.

In yet a further aspect of the invention,
recombinant myocytes are provided which may be used to
advantage in assessing the activity of agents that may
10 possess cardioprotective activity. Cardiac myocytes may
be transfected with any of the adenosine receptor
encoding cDNAs and used to screen for novel therapeutic
agents.

15 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figures 1A and 1B are graphs showing the effects of
adenosine A3 receptor antagonists on the
2-chloro-N^6-cyclopentyl-adenosine (CCPA) and
2-chloro-N^6-(3-iodobenzyl)adenosine- 5′-N-methyluronamide
20 (Cl-IB-MECA)-induced cardioprotective effect. Cultured
ventricular myocytes were prepared and the extent of
hypoxia-induced myocyte injury determined as described
hereinbelow. The A3 antagonist, 3-ethyl-
5-benzyl-2-methyl-6-phenyl
25 -4-phenylethynyl- 1,4-(1)-dihydropyridine-3,5-
dicarboxylate (MRS 1191) was present at the indicated
concentrations individually, with CCPA (10 nM), or with
Cl-IB-MECA (10 nM) during the ninety-minute hypoxia.
The percentage of myocytes killed, Figure 1A and the
30 amount of CK released, Figure, 1B, were determined
following the prolonged hypoxia. Data represent the
mean ±SE of three experiments.

Figure 2 is a graph showing effects of adenosine A1
35 agonists, 2-chloro-N^6-cyclopentyladenosine (CCPA),
N^6-cyclohexyladenosine (CHA), and adenosine amine
congener (ADAC) on cardiac myocyte injury in the

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presence or absence of excess 8-cyclopentyl-1,3-
dipropylxanthine (DPCPX), an A1 antagonist. (open
squares, CCPA; open triangles, CHA, open circles, ADAC;
filled squares CCPA + DPCPX; filled triangles, CHA +
5   DPCPX; filled circles, ADAC + DPCPX)

        Figures 3A-3F are graphs illustrating the
cardioprotective effects of the MRS compounds of the
invention. Figure 3A shows the amount of creatine
10   kinase released as a function of increasing
concentrations of MRS 584 in the presence (diamonds) or
absence (open squares) of the A1 receptor antagonist
DPCPX. Figure 3B shows the amount of creatine kinase
released as a function of increasing concentrations of
15   MRS 537 in the presence (diamonds) or absence (open
squares) of the A1 receptor antagonist DPCPX. Figure 3C
shows the amount of creatine kinase released as a
function of increasing concentrations of MRS 479 in the
presence (diamonds) or absence (open squares) of the A1
20   receptor antagonist DPCPX. Figure 3D shows the amount
of creatine kinase released as a function of increasing
concentrations of MRS 1340 in the presence (diamonds) or
absence (open squares) of the A1 receptor antagonist
DPCPX. Figure 3E shows that both MRS584 and MRS537 can
25   exert the cardioprotective effect of preconditioning via
the human adenosine A3 receptor. Data represent means
± SE of three experiments. Figure 3F shows that Cl-IB-
MECA reduces the percentage of myocytes killed during
ischemia.
30

        Figures 4A, and 4B are graphs showing the reduction
in cardiac myocyte cell death following simultaneous
exposure to A1 and A3 agonists on cardiac cells. Figure
4A shows that the preconditioning effect is
35   synergistically enhanced when adenosine A1 and A3
receptors are activated simultaneously, as opposed to
activation of a single receptor. The percentage of

myocytes killed is also significantly reduced in the
simultaneous presence of A1 and A3 agonists during
prolonged ischemia. See Figure 4B. Data represent
means ± SE of four experiments.

5

Figures 5A- 5D are graphs showing the
cardioprotective effects mediated by MRS 646 and MRS
1364 which activate the A1 and A3 receptors
simultaneously. Figure 5A shows that MRS646 and MRS1364
10   can pharmacologically precondition the cardiac myocyte.
The extent of myocyte injury was quantitated as
percentage of myocytes killed. The data show that when
either MRS646 or MRS1364 was present during the
injury-producing ischemia, both compounds protect
15   against injury. See Figure 5B. Figure 5C shows that
MRS 1364 was able to pharmacologically precondition the
cardiac myocytes transfected with the human adenosine A3
receptor. Data represent the means ± S.E. of three
experiments. Figure 5D shows that MRS 646 was able to
20   pharmacologically precondition the cardiac myocytes
transfected with the human adenosine A3 receptor. Data
represent the means ± S.E. of three experiments.

Figures 6A, 6B and 6C are graphs that show the
25   cardioprotective effects of MRS1543, a binary A1/A3
agonist. Figure 6A shows that the protective effect of
MRS1543 was only partially attenuated by DPCPX or by
MRS1191. Ventricular myocytes were exposed to the
indicated concentrations of MRS1543 in the presence or
30   the absence of excess DPCPX (1 μM) or excess MRS1191 (1
μM). The ventricular myocytes were also exposed to the
indicated concentrations of MRS1543 in the presence or
the absence of excess DPCPX (1 μM) plus excess MRS1191
(1 μM). See Figure 6B. The combined presence of both
35   DPCPX and MRS1191 completely abolished the protective
effect of MRS1543. The protective effect of MRS1543 in
chick cells transfected with the human adenosine A3

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receptor is shown in Figure 6C. Data represent means ± SE of three experiments.

Figures 7A and 7B are graphs showing the
5   cardioprotective effects of simultaneous administration of an A3/A1 agonist, MRS 580, and an A_{2a} antagonist on myocyte survival. Figure 7A shows that an A_{2a} antagonist enhances the ability of an A3/A1 agonist, MRS 580, to cause preconditioning. Figure 7B shows that an A_{2a}
10   antagonist enhances the protective effect of MRS 580 during prolonged ischemia. Figure 7C shows that MRS 580 can also precondition the cardiac myocyte via the human adenosine A3 receptor.

15   Figures 8A-8E are a series of graphs showing the cardioprotective effects of MRS1528, a binary conjugate having agonist activity at the A3 receptor and antagonist activity at the A2a receptors. Figure 8A shows that the ability of MRS1528 to cause
20   preconditioning is not affected by the presence of 8-(3-chlorostyryl)caffeine, (CSC). Myocytes were exposed to the indicated concentrations of MRS1528 in the presence or the absence of CSC (1 μM). Myocytes were also exposed to the indicated concentrations of MRS1528 in
25   the presence or the absence of CGS21680 (0.3 μM). See Figure 8B. Figure 8C shows the results obtained when myocytes were exposed to MRS1525 in the presence or the absence of CSC. Figure 8D shows the results obtained when myocytes were exposed to the indicated
30   concentrations of MRS1525 in the presence or the absence of CGS21680. The preconditioning effect of MRS1528 in cells transfected with the human adenosine A3 receptor is shown in Figure 8E. Data represent the means ± SE of three experiments.
35

Figure 9 is a diagram showing the synthetic scheme utilized to generate the binary compounds used in the

practice of this invention.

Figures 10A-10C depict diagrams for synthesizing certain compounds of the invention. Figures 10A and 10B show a synthetic scheme for synthesizing a derivative of an A1 selective agonist for coupling to an amine derived A3 agonist. Figure 10C is a schematic diagram showing the synthesis of binary conjugates with extended linkers.

Figures 11A and 11B show the nucleotide sequence of the cDNA encoding the adenosine A1 receptor.

Figures 12A-12D show the nucleotide sequence of the cDNA encoding the adenosine A2a receptor.

Figures 13A-13C show the nucleotide sequence of the cDNA encoding the adenosine A3 receptor.

DETAILED DESCRIPTION OF THE INVENTION

The cardioprotective roles of adenosine A1 and A3 receptors were investigated in a cardiac myocyte model of injury. The adenosine A3 receptor is a novel cardiac receptor capable of mediating potentially important cardioprotective functions. Prolonged hypoxia with glucose deprivation was used to simulate ischemia and to induce injury in cardiac ventricular myocytes cultured from chick embryos 14 days in ovo. When present during the prolonged hypoxia, the adenosine A3 agonists N6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA) and Cl-IB-MECA caused a dose-dependent reduction in the extent of hypoxia-induced injury, as manifested by a decrease in the amount of creatine kinase released and the percentage of myocytes killed. The adenosine A1 agonists CCPA and N6-cyclohexyladenosine (CHA) were also able to cause a decrease in the extent of myocyte injury. The A1

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receptor-selective antagonist DPCPX blocked the
cardioprotective effect of the A1 but not of the A3
agonists. Conversely, selective A3 antagonists MRS1191
and 3,5-Diethyl 2-methyl-6-phenyl-4-

5   [2-phenyl-(E)-vinyl]-1,4-(1)-dihydropyridine-3,5-dicarbo
xylate (MRS 1097) blocked the protection induced by
Cl-IB-MECA but had minimal effect on that caused by
CCPA. Thus, the cardioprotective effects of A1 and A3
agonists were mediated by their respective receptors.

10   The study identifies the cardioprotective function of
the cardiac A3 receptor and provides conclusive evidence
that simultaneous activation of both A1 and A3 receptors
during hypoxia can attenuate myocyte injury. This
finding is in contrast to that set forth by Downey et

15   al. in U.S. Patent No. 5,573,772 wherein administration
of antagonists to the A1 receptor in conjuction with
agonists at the A3 receptor was reported to enhance
cardioprotective effects during ischemia.
Administration of an adenosine A1 receptor antagonist is

20   not required for practicing the present invention.
       The present invention also includes administration
of binary reagents that selectively activate the A1 and
A3 adenosine receptors simultaneously. Concomitant
activation of the two receptors is believed to act

25   synergistically to enhance the cardioprotective effects
of preconditioning and to increase myocardial resistance
to ischemia.
       Several new MRS compounds are disclosed which
selectively activate the A3 adenosine receptor. See

30   Figures 3A-3F. These compounds are also contemplated
for use in the methods of the present invention.
       In accordance with another aspect of the invention,
a binary conjugate has been developed, which binds both
the A3 and A2a receptors simultaneously and elicits the

35   desired result, i.e., activation of the A3 receptor and
inhibition of the A2a receptor.
       A second binary conjugate, has also been

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synthesized in accordance with this invention which
simultaneously binds and activates both of the A1 and
the A3 receptors. These binary compounds may also be
used to advantage in practicing the methods of the
5 present invention. The following definitions are
provided to facilitate understanding of the present
invention.

Preconditioning ischemia – A brief ischemia which does
10 not cause any cardiac damage, but is able to protect the
heart against damage during a subsequent prolonged
ischemia. The effect of preconditioning ischemia is
mediated by adenosine, which is released during the
ischemia. Preconditioning may be induced by brief
15 exposure to anoxic conditions for example.

Adenosine receptors – A1, A3 and A2a receptors are
present on the myocardium (cardiac muscle cells). While
activation of the A1 and A3 receptors is
20 cardioprotective, activation of the A2a receptors is
deleterious and causes damage to the cardiac muscle
cells.

Stable angina – Condition observed in certain cardiac
25 patients having a chronic risk for mycardial ischemia
because of the chronic potential for an imbalance
between the supply of oxygenated blood and the demand
for it. Typically, such imbalance occurs during certain
stresses, such as exercise, emotional stress or stress
30 associated with a surgical procedure.

Unstable angina – Condition observed in cardiac patients
having frequent imbalance between the supply of and the
demand for oxygenated blood.
35

Post-myocardial infarction angina – Condition observed
in patients who have recurrent ischemia following a

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heart attack.

Preconditioning stimuli - Any drug, agent or treatment
which induces preconditioning, such as brief ischemia,
5   or A1 or A3 receptor agonists.

Myocardial responsiveness - The myocardium can be
treated so as to enhance the effectiveness and
protective effects of preconditioning. This enhancement
10   leads to a reduction in ischemic damage.

Representative examples of compounds used for practicing
the present invention are as follows
A1 agonists - CCPA, and compounds listed in Table I.
15   **A1 antagonists** - DPCPX
A3 agonists - IB-MECA, Cl-IB-MECA, MRS 584, MRS 537, MRS
479, MRS 1340, DBXMR, NNC21-0238, NN53-0055
A3 antagonists - MRS 1191
A2a agonists - CGS21680
20   **A2a antagonists** - CSC
Binary A3/A1 agonists - MRS 1543
Binary A3 agonist/A2a antagonists - MRS 1528
Mixed A3/A1 agonists - compounds listed in Table II,
including MRS 580 and MRS 1364
25   The present invention demonstrates that activation
of adenosine receptors during prolonged hypoxia can
attenuate the hypoxia-induced myocyte injury.
Specifically, activation of A1 and A3 receptors has been
shown to mediate adenosine-induced cardiac myocyte
30   protection. Additionally, the concomittant activation
of the A3 receptor coupled with inhibition of A2a
receptor activation by a selective binary conjugate has
also been shown to attenuate hypoxia-induced myocyte
injury. The following methods facilitate the practice
35   of the present invention.

Preparation of Cultured Ventricular Cells

Ventricular cells were cultured from chick embryos 14 days in ovo as previously described (16, 23). Cells were cultivated in a humidified 5% CO_2-95% air mixture at 37°C. All experiments were performed on day 3 in

5 culture, at which time cells exhibited rhythmic spontaneous contraction. The medium was changed to a HEPES-buffered medium containing (mM) 139 NaCl, 4.7 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 5 HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)

10 and 2% fetal bovine serum, pH 7.4, 37°C before exposing the myocytes to the various conditions at 37°C. Control myocytes were maintained in the HEPES-buffered media under room air. Ninety-minute exposure of the myocytes to hypoxia with glucose deprivation was used to induce

15 cell injury. Hypoxia was produced by placing the cells in a hypoxic incubator (NuAire) where O_2 was replaced by N_2 as previously described (16,23). Effects of adenosine receptor agonists and antagonists on the extent of myocyte injury were determined by exposure of the

20 prepared cells to these agents during the prolonged hypoxia.

Determination of Cell Injury

Determination of myocyte injury was made at the end

25 of the ninety-minute hypoxia, at which time myocytes were taken out of the hypoxic incubator and re-exposed to room air (normal % O_2). Aliquots of the media were then obtained for creatine kinase (CK) activity measurement, which is followed by quantitation of the

30 number of viable cells. Measurement of basal level of cell injury was made after parallel incubation of control cells under normal % O_2. The extent of hypoxia-induced injury to the ventricular cell was quantitatively determined by the percentages of cells

35 killed and by the amount of CK released into the media according to a previously described method (16, 23). Prior studies demonstrated that the cell viability assay

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distinguished the hypoxia-damaged from the control
normoxia-exposed cells. In brief, the media were
replaced with a trypsin-EDTA buffer to detach the cells,
which was then followed by sedimentation of the viable
5   myocytes. Parallel changes in % cells killed and CK
released (16, 23) further validated this assay for %
cells killed. The amount of CK was measured as enzyme
activity (unit/mg), and increases in CK activity above
the control level were determined. The percentage of
10   cells killed was calculated as the number of cells
obtained from the control group (representing cells not
subjected to any hypoxia or drug treatment) minus the
number of cells from the treatment group divided by
number of cells in control group multiplied by 100%.
15

**Synthesis of Adenosine A1 and A3 Receptor-selective
Agents** N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide
(IB-MECA), 2-chloro-N^6-(3-iodobenzyl)adenosine-
5'-N-methyluronamide (Cl-IB-MECA), and adenosine amine
20   congener (ADAC) were synthesized as previously described
(8,11,13). 3,5-Diethyl 2-methyl-
6-phenyl-4-[2-phenyl-(E)-vinyl]-1,4-(1)-
dihydropyridine-3,5-dicarboxylate (MRS 1097) and 3-ethyl
5-benzyl-2-methyl-6-phenyl-
25   4-phenylethynyl-1,4-(1)-dihydropyridine-3,5-
dicarboxylate (MRS 1191) were synthesized as previously
described (12).

Adenosine analogs
30   8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and
2-chloro-N^6-cyclopentyladenosine (CCPA),
N^6-cyclohexyladenosine (CHA) were from Research
Biochemicals International (Natick, MA). Embryonic chick
eggs were from Spafas Inc. (Storrs, Conn).
35

Transf ction of chick atrial myocyt s with the human A3
r c ptor - Atrial cells were cultured from chick embryos
14 days in ovo and transiently transfected with pcDNA3
(empty vector) or with pcDNA3/hA3R (full length cDNA
encoding human adenosine A3 receptor subcloned in
pcDNA3) using the calcium phosphate precipitation
method.   Forty-eight hours after transfection, the cells
were exposed to the compounds of the invention and the
percentage of myocytes killed during simulated ischemia
was determined.

     The following examples are provided to illustrate
certain embodiments of the invention. They are not
intended to limit the invention in any way.

EXAMPLE I
ACTIVATION OF A1 OR A3 ADENOSINE RECEPTORS REDUCES ISCHEMIC INJURY TO THE HEART

**A.   Exogenous adenosine causes a decrease in the extent
of cardiac myocyte injury during the prolonged hypoxia**
     Prolonged exposure to hypoxia in glucose-free media
induced significant cardiac myocyte injury with large
increase in the release of creatine kinase (28.5 ± 1.5
unit/mg, n= 4, ± SE) and in the % cells killed (30 ± 2
%, n= 4). Adenosine (10 μM), when added to the media
during the ninety-minute hypoxia, caused a decrease in
the amount of CK released (CK released in unit/mg = 14.9
± 3, n= 3) and in the % cells killed (% cells killed= 12
± 2 %, n= 4). This effect of exogenous adenosine was
blocked by the nonselective adenosine receptor
antagonist 8-sulfophenyl-theophylline (8-SPT, at 100 μM)
(in the presence of adenosine and 8-SPT: CK released in
unit/mg = 31 ± 5 and % cells killed = 28 ± 3 %, n=5).
The presence of 8-SPT during the hypoxia had no effect
on the level of myocyte injury (CK released= 29.6 ± 1.2
unit/mg; % cells killed= 31.6 ± 1.5 %, n=3). These data

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suggest that activation of adenosine receptors during
the prolonged hypoxia can protect the myocyte against
injury. Since adenosine can activate both the cardiac
A1 and A3 receptors and since 8-SPT, at 100 μM, can

5   block both receptors (23), the data are consistent with
the hypothesis that either receptor or both receptor
subtypes can mediate the cardioprotective function of
adenosine. This hypothesis was further explored using
selective agonists and antagonists. See Figures 1, 2

10   and 3.

**B. Adenosine A3 agonists mediate cardioprotective
effects during prolonged hypoxia**

In order to examine whether activation of adenosine

15   A3 receptors is capable of attenuating myocyte injury
during the prolonged hypoxia, agonists selective at the
A3 receptor were used. Prior study has demonstrated
that both IB-MECA and Cl-IB-MECA are highly selective at
the chick cardiac A3 receptor (23). Either A3 agonist,

20   when present during the prolonged hypoxia, was capable
of protecting the cardiac myocytes against
hypoxia-induced injury (Figure 1). The cardioprotective
effect of A3 agonists was quantitated as a decrease in
the amount of CK released and the percentage of myocytes

25   killed (statistically significant at 1 and 10 nM of
Cl-IB-MECA, ANOVA and t test, $p < 0.01$).

The presence of the A1 receptor-selective
antagonist DPCPX had no effect on the ability of IB-MECA
or Cl-IB-MECA to mediate their cardioprotective effects;

30   the A3 agonist-induced decreases in % cells killed and
CK released were similar in the presence and absence of
1 μM of DPCPX (not shown). These data indicate that the
cardioprotective effect of the A3 agonists was not due
to activation of the cardiac A1 receptor.

35   To determine whether the A3 agonist-induced
cardioprotection is mediated by the A3 receptor,
antagonists selective at the A3 receptor were employed.

Figure 1 demonstrates that the A3 receptor-selective antagonist MRS 1191 blocked the Cl-IB-MECA-induced cardioprotection. The levels of CK released and of percentage of cells killed were significantly higher in myocytes exposed to Cl-IB-MECA and 30 nM, 300 nM or 3 μM of MRS 1191 (ANOVA and t test, p<0.01). Another A3 receptor antagonist, MRS1097, was also able to block the cardioprotective effect of Cl-IB-MECA (not shown). These data provide conclusive evidence that activation of the A3 receptor can produce a potent cardioprotective effect when administered during the prolonged hypoxia.

C. Adenosine A1 receptor activation reduces cardiac myocyte injury during prolonged ischemia

Since the A1 receptor is also present on the cardiac myocyte, an investigation was undertaken to determine whether activation of the A1 receptor can confer a cardioprotective effect during the prolonged hypoxia. Prior study showed that CCPA, a known A1 agonist, is highly selective at the A1 receptor on these cardiac myocytes (23). Cultured ventricular myocytes were prepared and the extent of hypoxia-mediated myocyte injury determined. The various adenosine A1 receptor agonists were added to the media at the indicated concentrations in the absence or the presence of the A1 receptor antagonist DPCPX during the prolonged hypoxia. The percentage of cells killed was determined following the hypoxic exposure and removal of the A1 receptor agonists and antagonist. The data represent the mean of four experiments. At 1 and 10 nM of the A1 agonists, the percentages of myocytes killed were significantly lower than those obtained in the presence of either of the two A1 agonist concentrations and DPCPX (1 μM) (ANOVA and t test, P<0.01). Thus, CCPA caused a dose-dependent reduction in the percentage of myocytes killed as shown in Figure 2 and in the amount of CK released (not shown) (ANOVA and t test, p<0.01). Two

16

other A1 receptor-selective agonists, ADAC and CHA, were
also able to protect the myocytes when present during
the prolonged hypoxia. The cardioprotection stimulated
by CCPA, CHA and ADAC was blocked by the A1 antagonist

5   DPCPX. On the other hand, neither MRS 1191 nor MRS 1097
was able to block the CCPA-induced cardioprotection as
shown in Figure 1, providing definitive evidence that
the A1 agonist effect is mediated by the A1 receptor.

Although the number of viable cells was determined

10   quickly following re-exposure of cardiac myocytes to
normal % O_2 (reoxygenation), the A1 or the A3 agonist was
nevertheless present briefly prior to replacement with
the trypsin-EDTA buffer for cell viability assay. Thus,
it is possible that the decrease in myocyte injury is

15   due to the protection against a reoxygenation injury.
To study this possibility, CCPA or Cl-IB-MECA was added
immediately upon reoxygenation following the
ninety-minute hypoxic exposure. CCPA or Cl-IB-MECA was
maintained in the media for an additional hour prior to

20   determination of the percentage of myocytes killed.
Although CCPA or Cl-IB-MECA was able to protect the
myocytes when present during the reoxygenation, the
extent of protection was small (% myocytes killed
following the 90 minute hypoxia= 26.5 ± 1.0 %, n=6, ±

25   S.E. vs. CCPA present= 22.1 ± 1.5 %, n=5 or vs.
Cl-IB-MECA present= 23.0 ± 1.4 %, n=5; ANOVA and t
test, $P<0.05$) The previous example illustrates the
efficacy of A3/A1 adenosine receptor agonists in
reducing ischemic damage to the heart. A variety of

30   other compounds have been developed which also stimulate

17

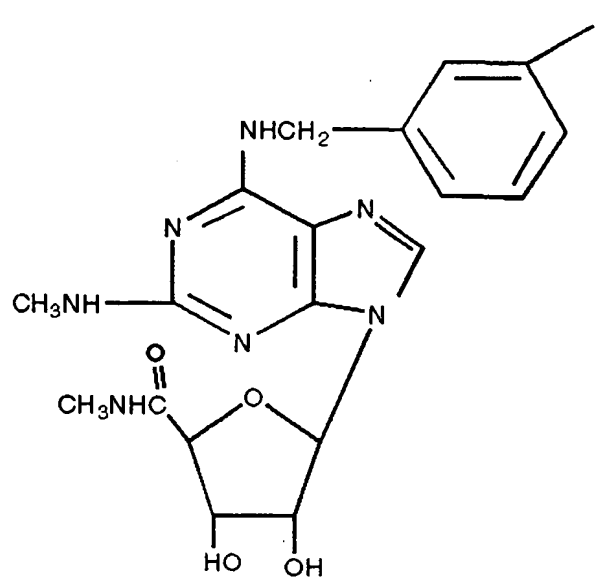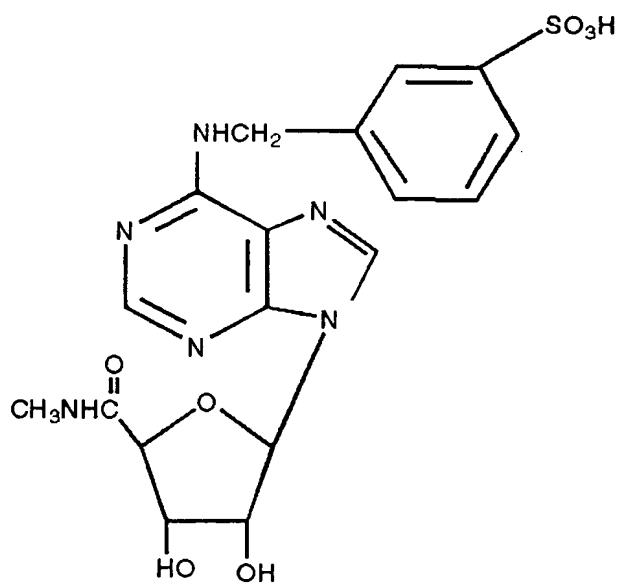A3 adenosine receptors. These are set forth below:
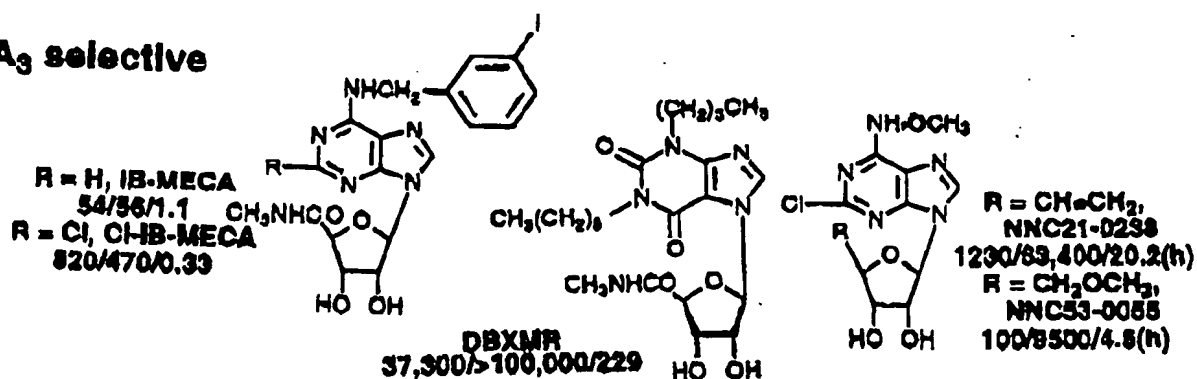
MRS 584

5

MRS 479

18
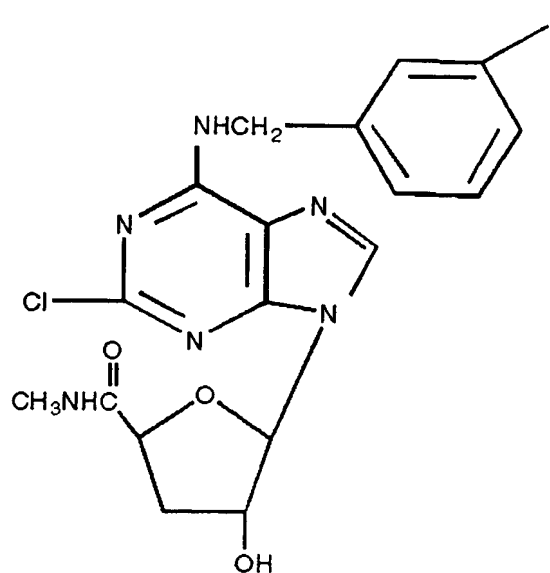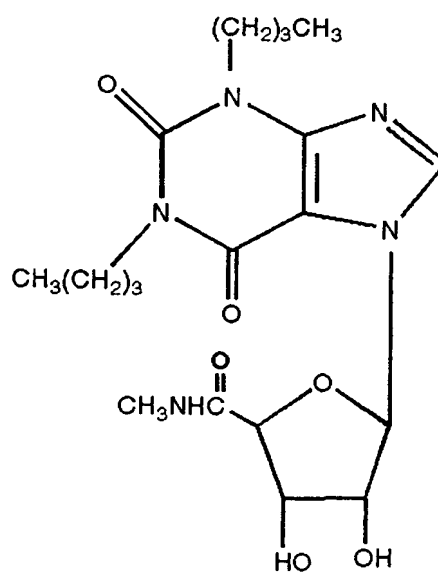
MRS 537

5

MRS 1340

Additional A3 selective agonists are set forth below:

5 **A₃ selective**

R = H, IB-MECA
54/56/1.1
R = Cl, Cl-IB-MECA
820/470/0.33

DBXRM
37,300/>100,000/229

R = CH=CH₂,
NNC21-0238
1290/83,400/20.2(h)
R = CH₂OCH₃,
NNC53-0055
100/8500/4.5(h)

10

15

The compounds shown immediately above have been
assayed for cardioprotective efficacy during prolonged
ischemia in the in vitro culture system described in
Example I.

20   The cardioprotective effects of A3 receptor
agonists MRS 584, MRS 537, MRS 479, MRS 1340 were
assessed in the presence or absence of the A1 receptor
antagonist DPCPX. The A1 receptor antagonist was
utilized to demonostrate the selectivity of the MRS

25   compounds for the A3 as opposed to the A1 adenosine
receptor. Figure 3A shows the cardioprotective effects
of MRS 584 in the presence and absence of DPCPX. The
extent of myocyte injury was plotted as the amount of
creatine kinase released during the prolonged simulat d

30   ischemia. Figure 3B shows the cardioprotective effects
of MRS 537 in the presence and absence of DPCPX. The

extent of myocyte injury was plotted as the amount of
creatine kinase released during the prolonged simulated
ischemia as a function of increasing the concentrations
of MRS 537. Figure 3C shows the cardioprotective

5   effects of MRS 479 in the presence and absence of DPCPX.
The extent of myocyte injury was plotted as the amount
of creatine kinase released during the prolonged
simulated ischemia as a function of increasing the
concentrations of MRS 479. Figure 3D shows the

10   cardioprotective effects of MRS1340 in the presence and
absence of DPCPX. The extent of myocyte injury was
plotted as the amount of creatine kinase released
during the prolonged simulated ischemia, as a function
of increasing the concentrations of MRS1340.

15   To determine whether the protective effects of the
compounds of the invention would also comparably
stimulate the human adenosine A3 receptor, atrial
cardiac myocytes, which express little if any endogenous
A3 receptor, were transfected with either empty vector

20   or vector containing the human adenosine A3 receptor
cDNA. The transfected myocytes were then exposed to the
compound for 5 minutes, which was followed by
replacement with fresh media and myocytes exposed to
normal conditions (non-ischemic) for 10 minutes prior to

25   being exposed to ninety minutes of ischemia. The
decrease in the number of myocytes killed in A3 receptor
cDNA-transfected myocytes as compared to
vector-transfected myocytes was plotted as a function of
the concentration of the compound. This decrease in

30   myocytes killed represented an index of the protection
mediated via the human adenosine A3 receptor.
            Figure 3E shows the protective effects of MRS 584
and MRS 537 obtained when chick cells were transfected
with a human A3 receptor encoded cDNA. These data show

35   that the MRS compounds of the invention specifically
bind to and activate the human A3 receptor.
            Figure 3F shows that chick atrial cells aquire A3-

21

receptor mediated cardioprotective responses upon
transfection with human A3 receptor encoding cDNA.
Cells were exposed to 10 nM Cl-IB-MECA and 1 μM DPCPX
for five minutes. Media were replaced with fresh media

5   lacking Cl-IB-MECA or DPCPX. Cells were then incubated
under room air for thirty minutes prior to being exposed
to 90 minutes of simulated ischemia. The graphs shows
the results obtained in cells transfected with A3
receptor encoding cDNA, untransfected cells and cells

10   transfected with empty vector. In untransfected cells
or cells transfected with empty vector, the A3 agonist
was able to reduce the percentage of cells killed when
compared to cells not pre-exposed to A3 agonist (control
cells, * t-test P<0.05). However, the A3 receptor

15   mediated reduction in the number of cells killed or the
amount of CK released, expressed as % decrease from
those obtained in the control cells, was significantly
greater in the cells transfected with the human A3
receptor encoding cDNA than in untransfected or cells

20   transfected with empty vector (one-way ANOVA and t test
P<0.01**)

     These data illustrate that these compounds
selectively activate human adenosine A3 receptor and can
be used to prevent ischemic damage to the heart.

25   Each of the above described MRS compounds is
contemplated for use in combination with any of the A1
agonists described herein for reducing ischemic damage
to the heart.

30

EXAMPLE II
ADENOSINE A1 AND A3 RECEPTOR AGONISTS ACT SYNERGISTICALLY TO INDUCE CARDIOPROTECTION.

35   Adenosine can exert two principal cardioprotective
effects. Adenosine can precondition the heart with
reduction in the size of myocardial infarction
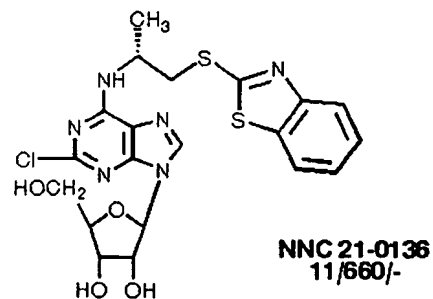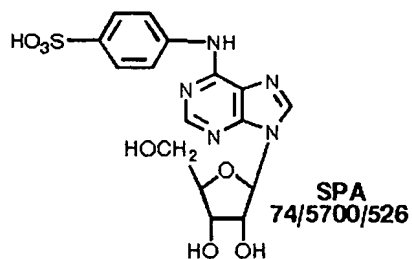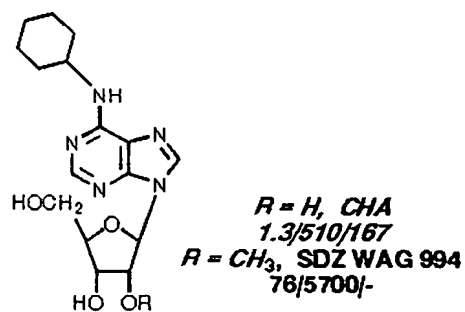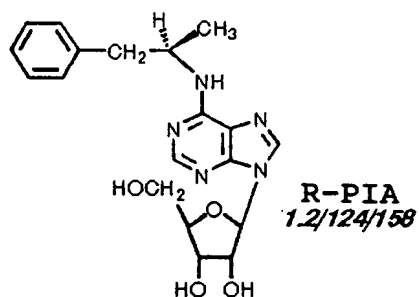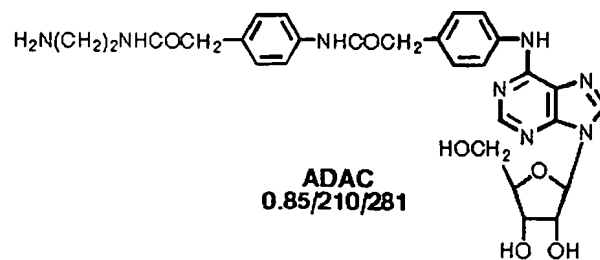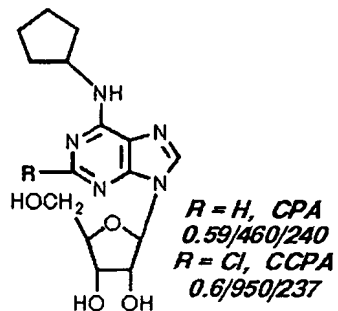(4,5,9,14,17,18). Intracoronary administration of

22

adenosine during reperfusion following prolonged no-flow
ischemia can also limit infarct size in the intact heart
(1, 19). Our previous studies have characterized a
cardiac myocyte model of injury, which is induced by
5   exposure of myocytes to a prolonged period of hypoxia in
glucose-free media (16, 23). Activation of either the
A1 or the A3 receptor by their respective agonists can
pharmacologically precondition the cardiac myocyte (23).
Figures 1 and 2 show that the presence of A1 or A3
10   agonist can also protect the cardiac myocytes when the
agonist is present individually during an actual
injury-inducing ischemia.

Figure 4A shows that the simultaneous activation of
both A1 and A3 receptors produces a greater
15   preconditioning effect than when either receptor is
activated separately. Thus, an A1 agonist and an A3
agonist interact synergistically to precondition the
cardiac myocyte. In addition, the simultaneous presence
of A1 and A3 receptor agonists during injury-producing
20   ischemia resulted in less myocyte injury than when only
A1 or A3 agonist is present. See Figure 4B. Thus,
simultaneous administration of A1 and A3 agonists will
lead to enhanced cardioprotection.

Based on the data presented above, it is apparent
25   that a variety of A1 agonists and A3 agonists may be
used in conjunction to reduce or prevent ischemic damage
to the heart. Suitable A1 agonists contemplated for use
in the present invention are set forth below in Table I.

30

TABLE I

A1 AGONISTS

A₁ selective

R = H, CPA
0.59/460/240
R = Cl, CCPA
0.6/950/237

ADAC
0.85/210/281

R-PIA
1.2/124/158

R = H, CHA
1.3/510/167
R = CH₃, SDZ WAG 994
76/5700/-

SPA
74/5700/526

NNC 21-0136
11/660/-

5

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EXAMPLE III
CARDIOPROTECTIVE EFFECT OF AGONISTS ACTIVATING
BOTH A1 AND A3 RECEPTORS

5

    In yet another embodiment of the invention, the
administration of an agonist capable of activating both
A1 and A3 receptors will be used in the methods of the
invention for reducing ischemic damage to the heart.

10 Referred to herein as a mixed A3/A1 agonist, these
agents mediate superior cardioprotective effects.
Examples of mixed A3/A1 agonists that may also be used
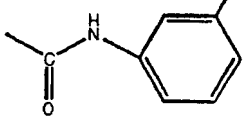in the practice of the present invention are listed in
Table II.

15

20
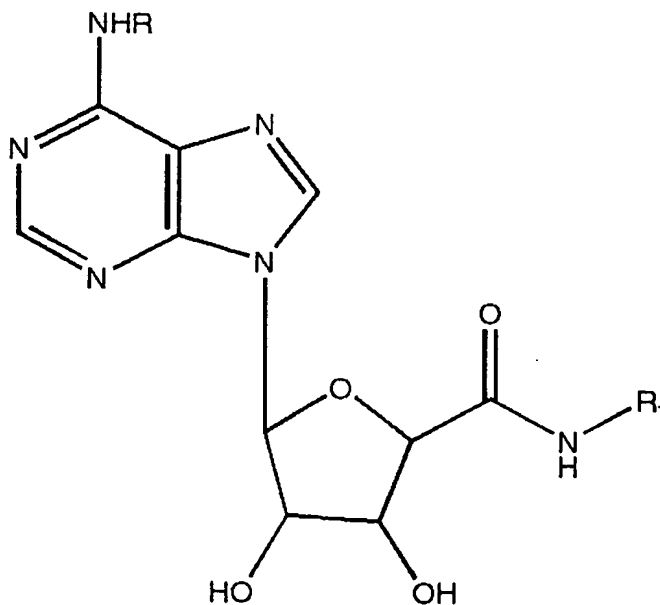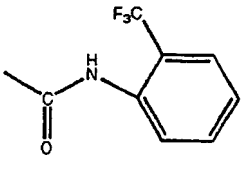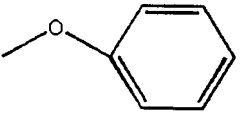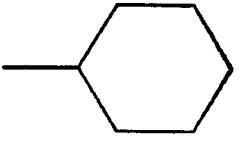
25

COMPOUND (Reference)	R_1	R	A_1 (nm)	A_{2a} (nm)	A_3 (nm)
Compound 4q (Reference 27) MRS 646	Ethyl	*(acetamido-3-iodophenyl structure)*	16	3940	30
Compound 4d (Reference 27)	Ethyl	*(acetamido-2-trifluoromethylphenyl structure)*	384	>10,000	54
Compound 37 (Reference 8)	Ethyl	*(4-nitro-ethylbenzyl structure)*	49	574	9.0
Compound 11 (Reference 28)	Methyl	*(methoxyphenyl structure)*	2060	66,300	1340
N^6-cyclohexyl NECA (Reference 29)	Ethyl	*(cyclohexyl structure)*	0.43	170	16

4q = N^6-((3-iodophenyl)carbamoyl)adenosine -5'uronamide

4d = N^6-((2-trifluoromethyl)carbamoyl)adenosine-5'uronamide

compound 37 = N^6-(4-Nitrobenzyl)adenosine-5'-N-ethyluronamide

compound 11 = 6-(O-Phenylhydroxylamino)purine-9-beta-ribofuranoside-5'-N-methyluronamide

N6-cyclohexyl NECA = N6-cyclohexyl 5'-N-ethylcarboxamidoadenosine

26
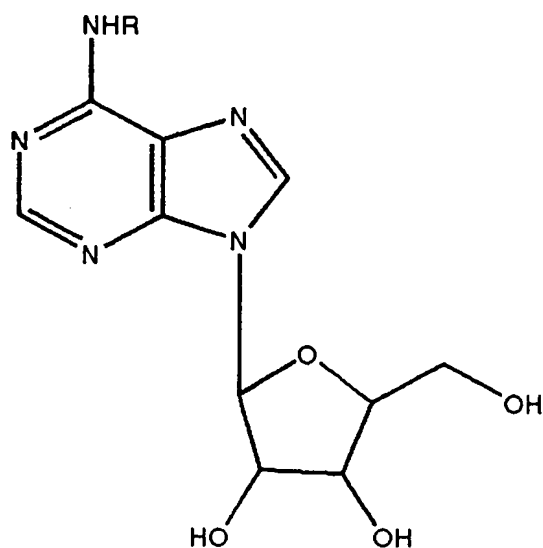
COMPOUND	R=	A_1 (nM)	A_2 (nM)	A_3 (nM)
Compound 8*	2-aminoethyl)amino]carbonyl]methyl]-anilino]carbonyl]methyl]phenyl	0.85	210	4

5

*N^6-[4-[[[4-[2-aminoethyl)amino]carbonyl]methyl]-anilino]carbonyl]methyl]phenyl]adenosine

10

Other examples of mixed A1/A3 agonists are MRS 580 and MRS 1364, the structures of which are shown below.

15

27

MRS 580

5
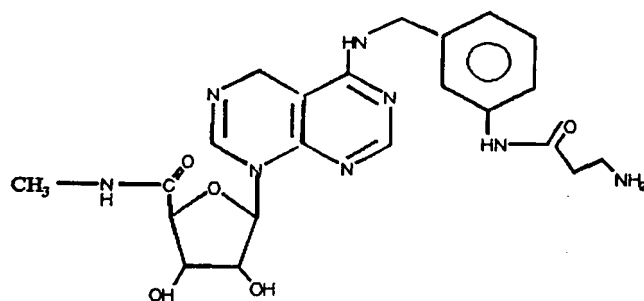
10
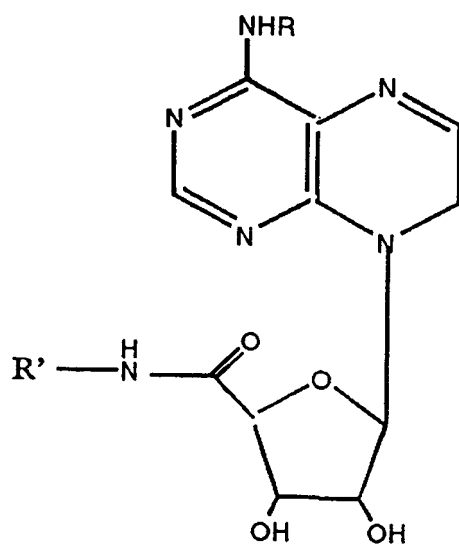
R'=Et

R = CONH-phenyl-NO$_2$

15   MRS 1364

20

25

28

Figure 5A shows the results obtained when
ventricular myocytes were exposed to the indicated
concentrations of MRS646 (structure 4Q, table II) or
MRS1364 for 5 minutes. Media were then replaced with
5   fresh media, which was then followed by exposure to
90-minute simulated ischemia and the data graphed. The
data show that these mixed A3/A1 agonists can
pharmacologically induce preconditioning in cardiac
myocytes.
10   Figure 5B shows that MRS 646 and MRS 1364 also
potently protect against myocyte injury during the
sustained simulated ischemia. Figures 5C and 5D show
that these agonists can pharmacologically precondition
the cardiac myocyte and induce potent cardioprotection
15   via the human adenosine A3 receptor.
In yet another embodiment of the invention, a
binary conjugate has also been synthesized which binds
and activates both the A1 and A3 receptors
simultaneously. Figure 6A shows that this binary
20   conjugate, MRS1543, can pharmacologically precondition
the cardiac myocyte and induce a potent cardioprotective
effect. The protective effect is only partially blocked
by A1 antagonist DPCPX; similarly, the protection is
only partially attenuated by the A3 antagonist MRS1191.
25   Figure 6B shows, however, that the combined presence of
DPCPX and MRS1191 completely abolished the protective
effect of MRS1543, indicating that the protection is
mediated via activation of both the A1 and the A3
receptors. Figure 6C shows that MRS1543 can
30   precondition and induces a cardioprotective effect via
the human adenosine A3 receptor.

35

40

EXAMPLE IV
SIMULTANEOUS ADMINISTRATION OF A3/A1 ADENOSINE RECEPTOR
AGONIST AND ADENOSINE A2a RECEPTOR
ANTAGONIST GIVES RISE TO ENHANCED CARDIOPROTECTION

5

In yet another embodiment of the invention the simultaneous administration of A3 and A1 adenosine receptor agonist and A2a antagonist is contemplated. Preferred A2a adenosine receptor antagonists for use in

10   the present invention are listed below in Table III:
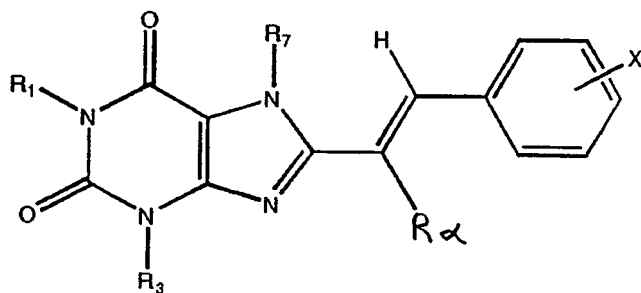
15

R1, R3 = methyl, ethyl, propyl, allyl

R7 = H, methyl, alkyl (C2-C8)

Rα = H (unless noted)

X   = is shown in Table III

30

TABLE III
Affinities of 8 Styrylxanthine Derivatives in
Radioligand Binding Assays at Rat Brain A_1 and A_2-Receptors (31)

cmpd	R_1, R_3	R_7	X	A_1/A_2 ratio
15b	Me	Me	H	41
17b	Me	Me	2-MeO	18
19b	Me	Me	3-MeO	64
20b	Me	Me	$3-CF_3$	25
21b	Me	Me	$3-NO_2$	11
22b	Me	Me	$3-NH_2$	30
23	Me	Me	3-(AcNH)	240
24	Me	Me	$3-(HOOC(CH_2)_2CONH)$	250
25	Me	Me	3-(t-BOC-NH)	30
26	Me	Me	$3-[(t-BOC)_2N]$	15
27b	Me	Me	3-F	190
28	Me	Me	3-Cl	520
29b	Me	Me	4-MeO	44
32b	Me	Me	$3,4-(MeO)_2$	70
33a	Me	H	$3,5-(MeO)_2$	25
33b	Me	Me	$3,5-(MeO)_2$	>200
34b	Me	Me	$3,5-F_2$	230
35	Me	Me	$3,5-(MeO)_2-4-OH$	19
36	Me	Me	$4-AcO-3,5-(MeO)_2$	93
37	Me	Me	$4-(4-PhCH_2O)-3,5-(MeO)_2$	30
38	Me	Me	$4-(4-NH_2-BuO)-3,5 (MeO)_2$	36
39	Me	Me	$4-[4-(tBOC-NH)BuO]-3,5-(MeO)_2$	42
40	Me	Me	$4-(4-NH_2-trans-CH_2CH= CHCH_2O-3,5-(MeO)_2$	28
41	Me	Me	$4-(4-AcNH-trans-CH_2CH= CHCH_2O)-3,5-(MeO)_2$	>50
42	Me	Me	$4-(4-t-BOC-NH-trans-CH_2CH= CHCH_2O-3,5-(MeO)_2$	>40
43b	Me	Me	$2,3,4-(MeO)_3$	34
44b	Me	Me	$3,4,5-(MeO)_3$	70 [>5600]
45b	Et	Me	$3,4,5-(MeO)_3$	34
46	allyl	Me	$3,4,5-(MeO)_3$	13 [>6700]
51b	Pr	Me	3-Cl	14
52b	Pr	Me	$3,4-(MeO)_2$	19 [190]
53b	Pr	Me	$3,5-(MeO)_2$	110

31

Additional compounds contemplated for use as A_{2a} antagonists include:
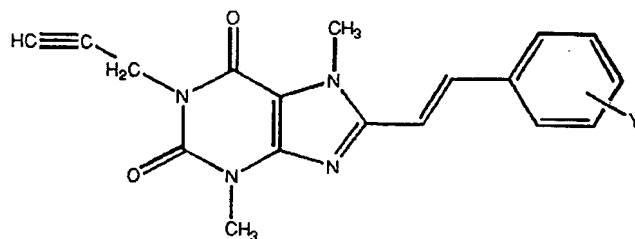
5   Y = m-Br or p-SO₃H   (DMPX)

10
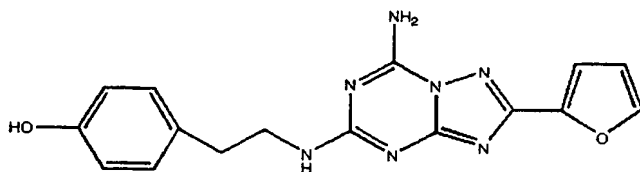
(ZM241385)

15

(SCH58261)

20

32

The results shown in Figures 7A and 7B indicate that
the simultaneous administration of an A3/A1 agonist and

5   an A2a antagonist gives rise to enhanced
cardioprotection. Cardiac myocytes were prepared as
described in Example I. The A1/A3 agonist, MRS 580, was
delivered in the presence or absence of the A2a
antagonist CSC. Data were plotted as the percentage of
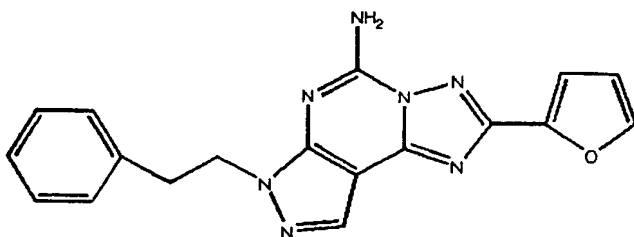
10   cells killed vs. the various drug combinations as
indicated. Data represent the means ± S.E. of three
experiments. Figure 7C shows that MRS 580 can
precondition the cardiac myocytes via the human adenosine
A3 receptor.

15   In another embodiment, a binary conjugate, MRS 1528,
was synthesized which acts as an agonist at the A3
receptor and an antagonist at the A2a receptor
simultaneously. Figure 8A shows that the protective
effect of MRS1528 was unaffected by the A2a receptor

20   antagonist CSC, consistent with the ability of MRS1528 to
block the A2a receptor. Figure 8B shows that the
protective effect of low concentrations of MRS1528 is
attenuated by the A2a agonist CGS21680 whereas the
protective effect of high concentrations of MRS1528 is

25   not affected by the presence of CGS21680. Together,
these data indicate that MRS1528 can activate A3 receptor
to induce preconditioning and can simultaneously block
the A2a receptor to enhance its preconditioning effect.
Further support for this concept comes from studies

30   testing the A3 agonist moiety of MRS1528, MRS1525.
MRS1525 does not contain the A2a antagonist moiety and in
response to the CSC, showed a uniform CSC-mediated
increase in the extent of preconditioning effect (Fig.
8C). In the concomitant presence of CGS21680, the

35   preconditioning effect of MRS1525 is attenuated at both
the high and low concentrations of MRS1525 (Fig. 8D).
Figure 8E shows that MRS1528 can cause preconditioning of

the cardiac myocytes via human adenosine A3 receptor.

A binary conjugate was synthesized with the general structure shown below. This conjugate binds both the A2a and the A3 receptors and acts as an agonist at the A3 receptor and an antagonist at the A2a receptor simultaneously. In the exemplary conjugate disclosed herein, MRS 1528, R = H and n =2.

A3/A2a binary conjugate
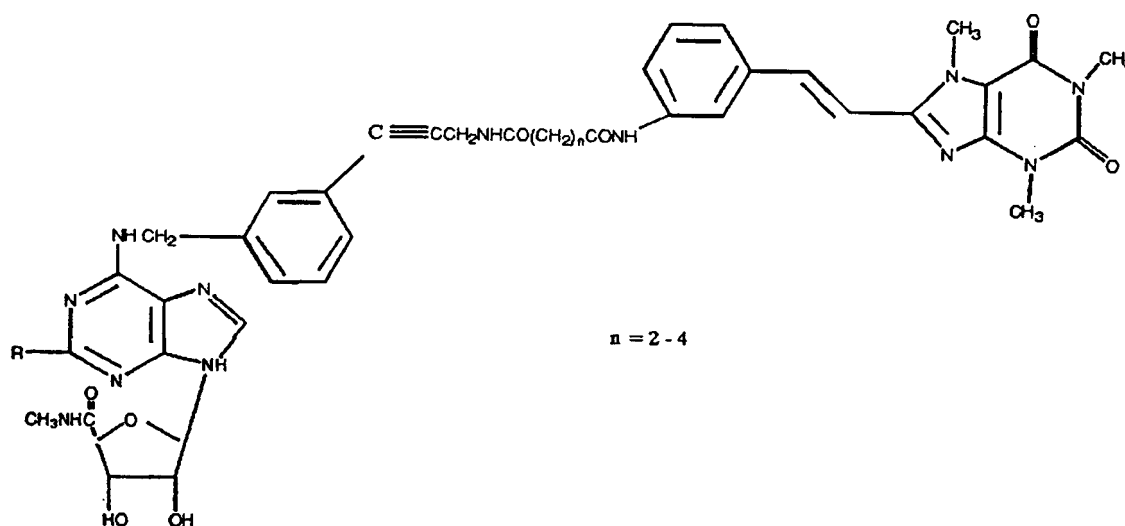
$$C\equiv CCH_2NHCO(CH_2)_nCONH$$

$$n = 2 - 4$$

A3/A2a Binary conjugate R = H, IB-MECA; R = Cl, Cl-IB-MECA

34

A second binary conjugate has also been synthesized which binds and activates both the A1 adenosine and A3 adenosine receptors simultaneously and has the general structure shown below. In an exemplary A1/A3 binary

5   conjugate of the invention, MRS 1543, R=H.
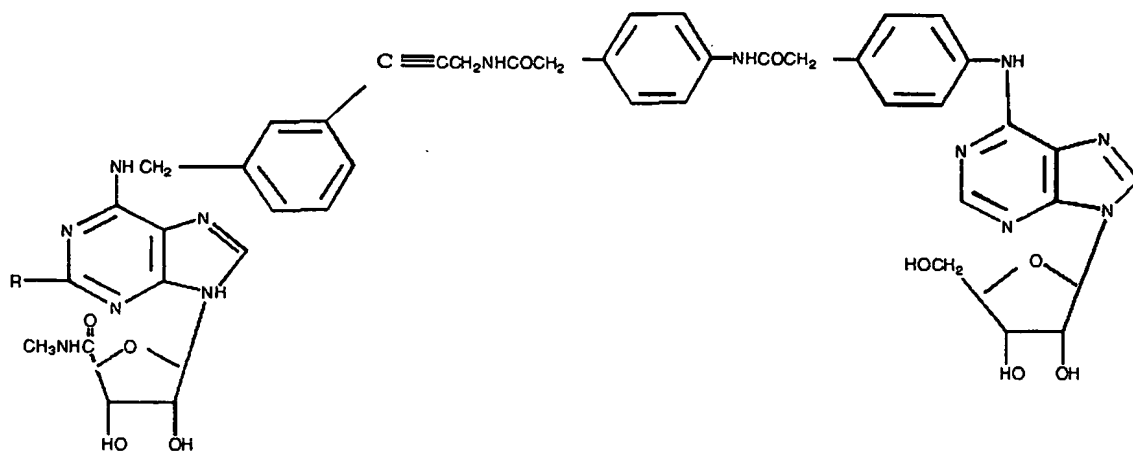
10   A3/A1 binary conjugate

     R = H, IB-MECA

     R = Cl, CL-IB-MECA

     Figure 9 sets forth an exemplary synthetic scheme utilized to produce the binary conjugate specific for the

15   A1 and A3 receptors.

     Figures 10A -10C depict schematic diagrams for synthesizing the compounds of the invention. Figures 10A-10B shows a synthetic scheme for generating a derivative of an A1 selective agonist for coupling to an

20   amine derived A3 agonist. Figure 10C shows a synthetic scheme for conjugating the reagents via an extended linker. Extended linkers may increase the affinity and potency of the conjugates at the adenosine receptor. Table IV lists the names of the chemical structures

25   appearing in Figures 9, 10B and 10C.

35

TABLE IV

IB-MECA = N^6-(3-iodobenzyl)-5'-N-
methylcarboxamidoadenosine

Compound III = N^6-[3-(3-amino-1-propynyl)benzyl]-5'-
N-methylcarboxamidoadenosine

Compound VIII (m- and p-isomers) = N^6-[3-[3-(3-
isothiocyanatophenylaminothiocarbonyl)-amino-1-
propynyl]benzyl]-5'-N-
methylcarboxamidoadenosine
and
N^6-[3-[3-(4-isothiocyanatophenylaminothiocarbonyl)-
amino-1-propynyl]benzyl]-5'-N-
methylcarboxamidoadenosine

ADAC = N^6-[4-[[[4-[[[(2-
aminoethyl)amino]carbonyl]methyl]anilino]carbon
yl]methyl]phenyl]adenosine

Compound IX (m- and p-isomers) = conjugate of N^6-
[4-[[[4-[[[(2-amino-
ethyl)amino]carbonyl]methyl]anilino]carbonyl]me
thyl]phenyl]adenosine and N^6-[3-[3-(3-
isothiocyanatophenylaminothiocarbonyl)-amino-1-
propynyl]benzyl]-5'-N-
methylcarboxamidoadenosine
and
conjugate of N^6-[4-[[[4-[[[(2-amino-
ethyl)amino]carbonyl]methyl]anilino]carbonyl]me
thyl]phenyl]adenosine and N^6-[3-[3-(4-
isothiocyanatophenylaminothiocarbonyl)-amino-1-
propynyl]benzyl]-5'-N-
methylcarboxamidoadenosine

Compounds XI =

MRS 1576 = t-Boc-L-alanyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-
methylcarboxamidoadenosine

MRS 1574 = t-Boc-L-methionyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-
methylcarboxamidoadenosine

MRS 1568 = t-Boc-L-valyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-
methylcarboxamidoadenosine

MRS 1571= t-Boc-L-leucyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-
methylcarboxamidoadenosine

MRS 1572 = t-Boc-L-isoleucyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-

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methylcarboxamidoadenosine

MRS 1573 = t-Boc-L-phenylalanyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-
methylcarboxamidoadenosine

Compounds XII =

MRS 1577 = L-alanyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-
methylcarboxamidoadenosine

MRS 1575 = L-methionyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-
methylcarboxamidoadenosine

MRS 1570 = L-valyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-
methylcarboxamidoadenosine

MRS 1569= L-leucyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-
methylcarboxamidoadenosine

MRS 1560= L-isoleucyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-
methylcarboxamidoadenosine

MRS 1565 = L-phenylalanyl-N^6-[3-(3-amino-1-
propynyl)benzyl]-5'-N-
methylcarboxamidoadenosine

Compound IV = N^6-[4-[[[4-
(carboxymethyl)anilino]carbonyl]methyl]phenyl]a
denosine

Compound XIII = conjugate of N^6-[4-[[[4-
(carboxymethyl)anilino]anilino]carbonyl]methyl]
phenyl]adenosine
and compound XII

Compound XIV = N^6-[4-(carboxymethyl)phenyl]adenosine

Compound XV = conjugate of N^6-[4-
(carboxymethyl)phenyl]adenosine
and compound XII

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EXAMPLE V
SCREENING ASSAY FOR IDENTIFYING COMPOUNDS THAT HAVE AFFINITY FOR THE A3 ADENOSINE RECEPTOR.

5

The recombinant cardiac atrial cells described above provide a system for assessing agents that may have cardioprotective activity. A generalized method for such screening would entail providing a fixed concentration of
10   a test compound to transfected cells and assessing whether or not the test compound exerted a protective effect during ischemia. Negative controls would comprise both untranfected cells and transfected cells not exposed to the test compound.

15   Once a test compound is determined to have cardioprotective activity, it will be serially diluted and applied to the transfected myocytes described above. In this way, the minimally effective concentration of the compound will be determined.

20   While $CaPO_4$ transfection is exemplified herein, the myocytes of the invention may be transfected using any method known to those of skill in the art. Such methods include, but are not limited to lipofectin, electroporation, or viral vector mediated transfection.

25   Cardiac myocytes may be transfected with any of the adenosine receptor having a known DNA sequence. Thus the assay is not limited to cells transfected with A3 encoding cDNA. Any of the known adenosine receptors may be transfected and assayed as described above. Figure 11
30   shows the nucleotide sequence of the cDNA encoding the A1 adenosine receptor. Figure 12 shows the nucleotide sequence of the cDNA encoding the A2a receptor and Figure 13 shows the sequence of the cDNA encoding the A3 receptor (32-34).

35

EXAMPLE VI
ADMINSTRATION MODALITIES SUITABLE
FOR THE COMPOUNDS OF THE PRESENT INVENTION

5   The protective effect of A3/A1 agonists has been
demonstrated herein in animal models. A1/A3 agonists may
be used therapeutically in patients who suffer from
ischemic damage due to stable angina, unstable angina or
post-myocardial infarction angina.

10   Several administration modalities may be utilized to
treat patients with the agonists and antagonists of the
invention. These modalities are influenced by
bioavailability factors. For example, if the compound is
metabolized in the liver or excreted in the bile, some of

15   the active compound absorbed from the gastrointestinal
tract will be inactivated by the liver before it can
reach the general circulation for distribution to the
site of action. It is not believed that the compounds of
the invention will be subject to this first pass loss.

20   Additionally, because the agonists of the invention are
polar and water soluble, it is expected that they will
have a small volume of distribution, and thus be readily
eliminated by the kidney. Moreover, binding of the
agonists to plasma proteins may limit their free

25   concentrations in tissues and at their locus of action
since it is only the unbound drug which equilibrates
across membrane receptor sites.

      Another factor affecting bioavailability is the
distribution of the agonists to tissues. The agonists of

30   the invention do not cross the blood brain barrier.
Given the relatively small size of the compounds and
their water solubility, it is anticipated that the
compounds will have a relatively fast second phase of
drug distribution. This distribution is determined by

35   both the blood flow to the particular tissue of the organ
such as the heart, as well as the rate at which the
compounds diffuse into the interstitial compartment from

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the general circulation through the highly permeable
capillary endothelium.

Patients may be perfused with the agonists of the
invention by dissolving them in normal saline solution or
using emulsifying agents or cosolvents followed by
intravenous administration every four to six hours.
Effective doses usually range from 100 to 300 nM. For
example, considering a 15 liter volume of distribution
for a 70 kg patient, a loading dose ranging from 0.5 to
1.5 mg is preferably used. Depending on the half-life of
the agonists in the body, several doses, e.g., 1.5-4.5 mg
may be adminstered per day.

Alternatively, a time-release or slow-release
preparation may be utilized which allows for periodic or
constant release of the antagonists over a given time
period. This method would allow for a single dose of the
agonists in a given day. Methods for preparing such
capules are well known to those of skill in the art of
drug delivery.

In summary, the present data illustrates the novel
synergistic protective function of simultaneous cardiac
A1 and A3 receptor activation. In addition to the
synergistic role in mediating preconditioning of the
cardiac myocyte, the data provide conclusive evidence
that activation of both receptors can also act
synergistically to attenuate myocyte injury during the
prolonged injury-producing ischemia. Thus, agonists
selective at the A1 and the A3 receptors represent novel
potent cardioprotective agents even when ischemia has
already begun. The concomitant administration of A1/A3
agonist with an A2a antagonist may also enhance
cardioprotection as will binary conjugate capable of
activating the A1 or A3 receptor while simultaneously
blocking the A2a receptor. These data have important
clinical implications in the treatment of ischemic heart
disease and implicate A1 and A3 receptor-selective
agonists for the reduction of the size of myocardial

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infarction when given during the infarct-producing
ischemia.

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10

     While certain preferred embodiments of the present
     invention have been described and specifically
     exemplified above, it is not intended that the invention
15   be limited to such embodiments. Various modifications may
     be made to the invention without departing from the scope
     and spirit thereof as set forth in the following claims.

What is claimed is:

1.   A method for preventing or reducing ischemic
damage to the heart, in a patient in need thereof,
comprising administering to said patient an agonist
having affinity for both the A1 and A3 adenosine
receptors in an amount effective to activate A3 and A1
receptors in the heart of said patient.

2.   A method as claimed in claim 1, wherein said
agonist is delivered using an administration means
selected from the group consisting of intravenous
administration, oral administration and cardiac
perfusion.

3.   A method as claimed in claim 1, wherein said
agonist is selected from the group of compounds listed in
Table II.

4.   A method as claimed in claim 1, wherein said
agonist is N^6-((2-trifluoromethyl)carbamoyl)
adenosine-5'uronamide.

5.   A method as claimed in claim 1, wherein said
agonist is N^6-((3-iodophenyl)carbamoyl)
adenosine-5'uronamide.

6.   A method as claimed in claim 1 wherein said
agonist is a binary conjugate which has affinity for, and
activates the A1 and A3 adenosine receptors
simultaneously.

7.   A method as claimed in claim 1 wherein said
agonist is administered to said patient prior to a
surgical procedure having potential to cause cardiac
ischemic damage.

8.   A method as claimed in claim 1, wherein said
agonist is administered to said patient during a surgical
procedure having potential to cause cardiac ischemic
damage.

5

9.   A method as claimed in claim 1, wherein said
agonist is administered to said patient following a
surgical procedure having potential to result in cardiac
ischemic damage.

10

10.   A method as claimed in claim 1, wherein said
patient is in need of said treatment due to an anginal
condition selected from the group consisting of chronic
stable angina, unstable angina, post myocardial

15   infarction angina.

11.   A method as claimed in claim 1, wherein said
patient is in need of such treatment due to acute
myocardial infarction.

20

12.   A method for preventing or reducing ischemic
damage to the heart, in a patient in need thereof,
comprising administering to said patient an mixed agonist
having affinity for the A3 and A1 adenosine receptors and

25   an antagonist having affinity for the A2a adenosine
receptor in amounts effective to activate said A3 and A1
receptors and inhibit activation of said A2a receptor in
the heart of said patient.

30   13.   A method as claimed in claim 12, wherein said
agonist and said antagonist are delivered using an
administration means selected from the group consisting
of intravenous administration, oral administration and
cardiac perfusion.

35

14.   A method as claimed in claim 12, wherein said
agonist is selected from the group of compounds listed in

Table II.

15. A method as claimed in claim 12 wherein said antagonist is selected from the group of compounds listed
5   in Table III.

16. A method as claimed in claim 12, wherein said agonist is N^6-((2-trifluoromethyl)carbamoyl) adenosine-5'uronamide.
10

17. A method as claimed in claim 12, wherein said agonist is N^6-((3-iodophenyl)carbamoyl)adenosine-5'uronamide.
15

18. A method as claimed in claim 12, wherein said agonist is selected from the group consisting of MRS 584, MRS 479, MRS 537 or MRS 1340.

20   19. A method as claimed in claim 12, wherein said antagonist is selected from the group consisting of CSC, DMPX, ZM241385 or SCH58261.

20. A method as claimed in claim 12, wherein said
25   agonist and said antagonist are administered to said . patient prior to a surgical procedure having potential to cause cardiac ischemic damage.

21. A method as claimed in claim 12, wherein said
30   agonist and said antagonist are administered to said patient during a surgical procedure having potential to cause cardiac ischemic damage.

22. A method as claimed in claim 12, wherein said
35   agonist and said antagonist are administered to said patient following a surgical procedure having potential to result in cardiac ischemic damage.

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23.   A method as claimed in claim 12, wherein said patient is in need of said treatment due to an anginal condition selected from the group consisting of chronic stable angina, unstable angina, and post myocardial infarction angina.

5

24.   A method as claimed in claim 12, wherein said patient is in need of said treatment due to acute myocardial infarction.

10

25.   A method for preventing or reducing ischemic damage to the heart, in a patient in need thereof, comprising administering to said patient a binary conjugate, which acts as an adenosine A3 receptor agonist while simultaneously inhibiting the activation of A2a receptors in an amount effective to enhance myocardial response to said preconditioning stimuli.

15

26.   A method as claimed in claim 25, wherein said patient is in need of such treatment due to a cardiac condition selected from the group consisting of chronic stable angina, unstable angina, post-myocardial infarction angina or acute myocardial infarction.

20

27.   A method as claimed in claim 25 wherein said agonist is administered to said patient prior to a surgical procedure which may cause cardiac ischemic damage.

25

28.   A method as claimed in claim 25, wherein said agonist is administered to said patient during a surgical procedure having potential to cause cardiac ischemic damage.

30

29.   A method as claimed in claim 25, wherein said agonist is administered to said patient following a surgical procedure which may result in cardiac ischemic

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damage.

30.   A method for preventing or reducing ischemic damage to the heart, in a patient in need thereof, comprising administering to said patient both an adenosine A3 receptor agonist and at least one adenosine A1 receptor agonist in an amount effective to activate the A1 and A3 adenosine receptors in the heart of said patient.

31.   A method as claimed in claim 30, wherein said agonists are delivered using an administration means selected from the group consisting of intravenous administration, oral administration and cardiac perfusion.

32.   A method as claimed in claim 30, wherein said agonist and said agonists are administered to said patient prior to a surgical procedure having potential to cause cardiac ischemic damage.

33.   A method as claimed in claim 30, wherein said agonist and said antagonist are administered to said patient during a surgical procedure having potential to cause cardiac ischemic damage.

34.   A method as claimed in claim 30, wherein said agonist and said antagonist are administered to said patient following a surgical procedure having potential to result in cardiac ischemic damage.

35.   A method as claimed in claim 30, wherein said patient is in need of said treatment due to an anginal condition selected from the group consisting of chronic stable angina, unstable angina, and post myocardial infarction angina.

36. A method as claimed in claim 30, wherein said patient is in need of said treatment due to acute myocardial infarction.

5   37. A method as claimed in claim 30, wherein said A3 agonist is selected from the group of compounds consisting of IB-MECA, Cl-IB-MECA, MRS 584, MRS 479, MRS 537, MRS 1340 and DBXMR and said A1 agonist is selected from the group of compounds listed in Table I.

10

38. A binary conjugate for preventing or reducing ischemic damage to the heart, said conjugate acting as an agonist at the A3 adenosine receptor and an antagonist at

15   the A2a adenosine receptor.

39. A binary conjugate as claimed in claim 38, said conjugate having the structure of MRS 1528.

20   40. A method for administering the binary conjugated of claim 38, wherein said conjugate is delivered using an administration means selected from the group consisting of intravenous administration, oral administration and cardiac perfusion.

25

41. A method as claimed in claim 40, wherein said conjugate is administered to said patient prior to a surgical procedure having potential to cause cardiac ischemic damage.

30

42. A method as claimed in claim 40, wherein said conjugate is administered to said patient during a surgical procedure having potential to cause cardiac ischemic damage.

35

43. A method as claimed in claim 40, wherein said conjugate is administered to said patient following a

surgical procedure having potential to result in cardiac
ischemic damage.

44. A method as claimed in claim 40, wherein said
5   patient is in need of said treatment due to an anginal
condition selected from the group consisting of chronic
stable angina, unstable angina, and post myocardial
infarction angina.

10   45. A method as claimed in claim 40, wherein said
patient is in need of said treatment due to acute
myocardial infarction.

15   46. A binary conjugate for preventing or reducing
ischemic damage to the heart, said conjugate acting as an
agonist at the A3 adenosine receptor and an agonist at
the A1 adenosine receptor.

20   47. A binary conjugate as claimed in claim 46, said
conjugate being selected from the group of compounds
consisting of MRS1543, a conjugate of N^6-[4-[[[4-
(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl]
adenosine and compound XII of Figure 10B, wherein R' on
25   compound XII is H, a conjugate of N^6-[4-[[[4-
(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl]
adenosine and compound XII of Figure 10B, wherein R' on
compound XII is Ch_3, a conjugate of N^6-[4-[[[4-
(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl]
30   adenosine and compound XII of Figure 10B, wherein R' is
$(CH_2)_2SCH_3$, a conjugate of N^6-[4-[[[4-
(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl]
adenosine and compound XII of Figure 10B, wherein R' is
$CH(CH_3)_2$, a conjugate of N^6-[4-[[[4-
35   (carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl]
adenosine and compound XII of Figure 10B, wherein R' is
$CH_2CH(CH_3)_2$, a conjugate of N^6-[4-[[[4-

(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl]
adenosine and compound XII of Figure 10B, wherein R' is
$C(CH_3)CH_2CH_3$, and a conjugate of N^6-[4-[[[4-
(carboxymethyl)anilino]anilino]carbonyl]methyl]phenyl]

5   adenosine and compound XII of Figure 10B, wherein R' is
$CH_2C_6H_5$.

48.   A binary conjugate as claimed in claim 46, said

10   conjugate being selected from the group of compounds
consisting of a conjugate of N^6-[4-(carboxymethyl)
phenyl]adenosine and compound XII of Figure 10B, wherein
R' is H, a conjugate of N^6-[4-(carboxymethyl)
phenyl]adenosine and compound XII of Figure 10B, wherein

15   R' is CH_3, a conjugate of N^6-[4-(carboxymethyl)
phenyl]adenosine and compound XII of Figure 10B, wherein
R' is $(CH_2)_2SCH_3$, a conjugate of N^6-[4-(carboxymethyl)
phenyl]adenosine and compound XII of Figure 10B, wherein
R' is $CH(CH_3)_2$, a conjugate of N^6-[4-(carboxymethyl)

20   phenyl]adenosine and compound XII of Figure 10B, wherein
R' is $CH_2CH(CH_3)_2$, a conjugate of N^6-[4-(carboxymethyl)
phenyl]adenosine and compound XII of Figure 10B, wherein
R' is $C(CH_3)CH_2CH_3$, and a conjugate of N^6-[4-
(carboxymethyl) phenyl]adenosine and compound XII of

25   Figure 10B, wherein R' is $CH_2C_6H_5$.

49.   A method for administering the binary
conjugated of claim 46, wherein said conjugate is
delivered using an administration means selected from the

30   group consisting of intravenous administration, oral
administration and cardiac perfusion.

50.   A method as claimed in claim 49, wherein said
conjugate is administered to said patient prior to a

35   surgical procedure having potential to cause cardiac
ischemic damage.

51.   A method as claimed in claim 49, wherein said
conjugate is administered to said patient during a
surgical procedure having potential to cause cardiac
ischemic damage.

5

52.   A method as claimed in claim 49, wherein said
conjugate is administered to said patient following a
surgical procedure having potential to result in cardiac
ischemic damage.

10

53.   A method as claimed in claim 49, wherein said
patient is in need of said treatment due to an anginal
condition selected from the group consisting of chronic
stable angina, unstable angina, and post myocardial

15   infarction angina.

54.   A method as claimed in claim 49, wherein said
patient is in need of said treatment due to acute
myocardial infarction.

20

55.   A recombinant cardiac myocyte comprising a
nucleic acid encoding an adenosine receptor selected from
the group consisting of the A3 receptor, the A3 receptor,

25   or the A2a adenosine receptor.

56.   A recombinant myocyte as claimed in claim 55,
whrerein the myocyte is a chick embryo ventricular
myocyte and the adenosine receptor is a human adenosine

30   receptor.

57.   A method for determining whether a test
compound exerts a cardioprotective effect, comprising:
       a)   providing a recombinant myocyte expressing

35   an adenosine receptor;
       b)   contacting said cells with said test
compound;

53

c) exposing cells to ischemic conditions; and

d) assessing the presence of said cardioprotective effect, if any, exerted by said test compound.

5

58. A method as claimed in claim 57, wherein said cardioprotective effect is assessed by determining the number of myocytes killed.

10   59. A method as claimed in claim 57, wherein said cardioprotective effect is assessed by determining the amount of creatine kinase released from said recombinant cardiac myocytes.

15   60. A method as claimed in claim 57, wherein said recombinant myocyte is selected from the group consisting of chick embryo ventricular myocytes or adult rat ventricular myocytes.

20   61. A method as claimed in claim 57, wherein said adenosine receptor is the human A3 adenosine receptor.

62. A method as claimed in claim 57, wherein said adenosine receptor is the human A1 adenosine receptor.

25

63. A method as claimed in claim 57, wherein said adenosine receptor is the human A2a adenosine receptor.

Fig. 1A
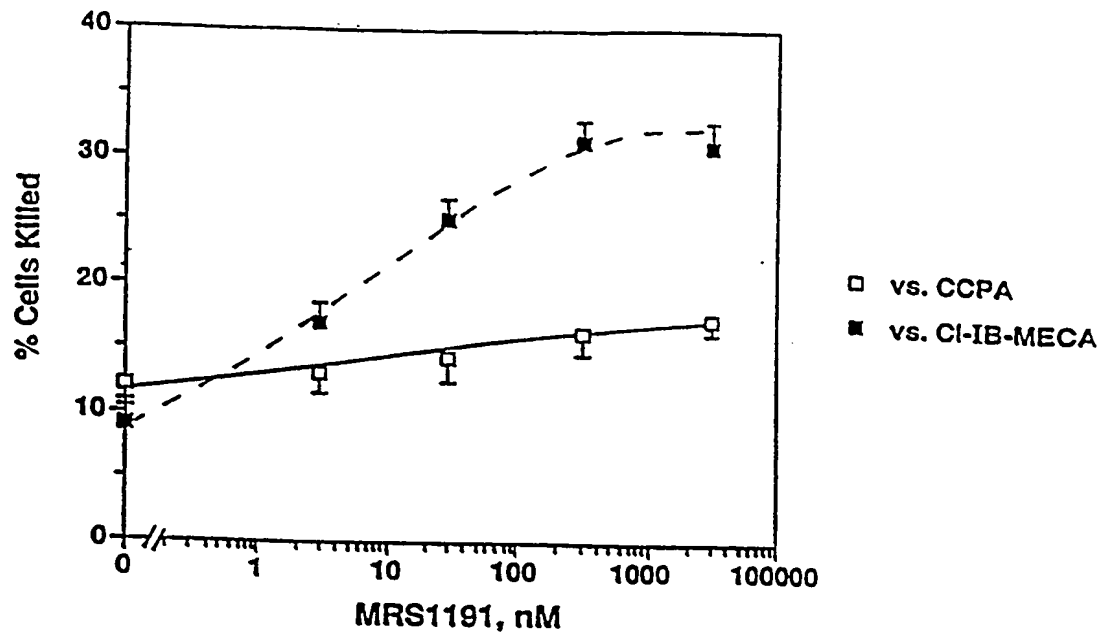
Fig. 1B
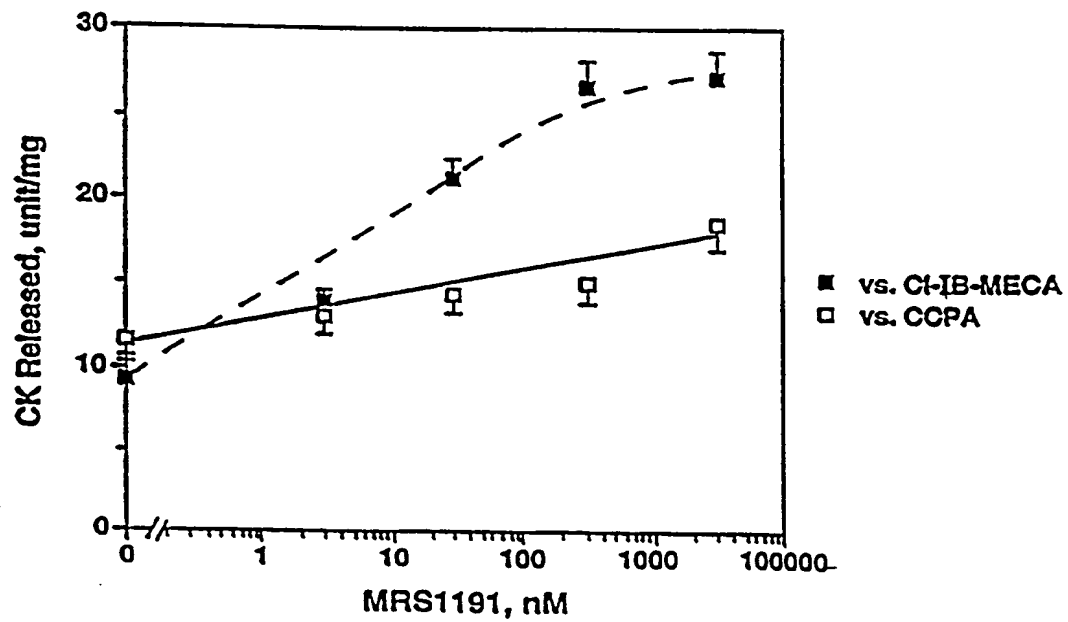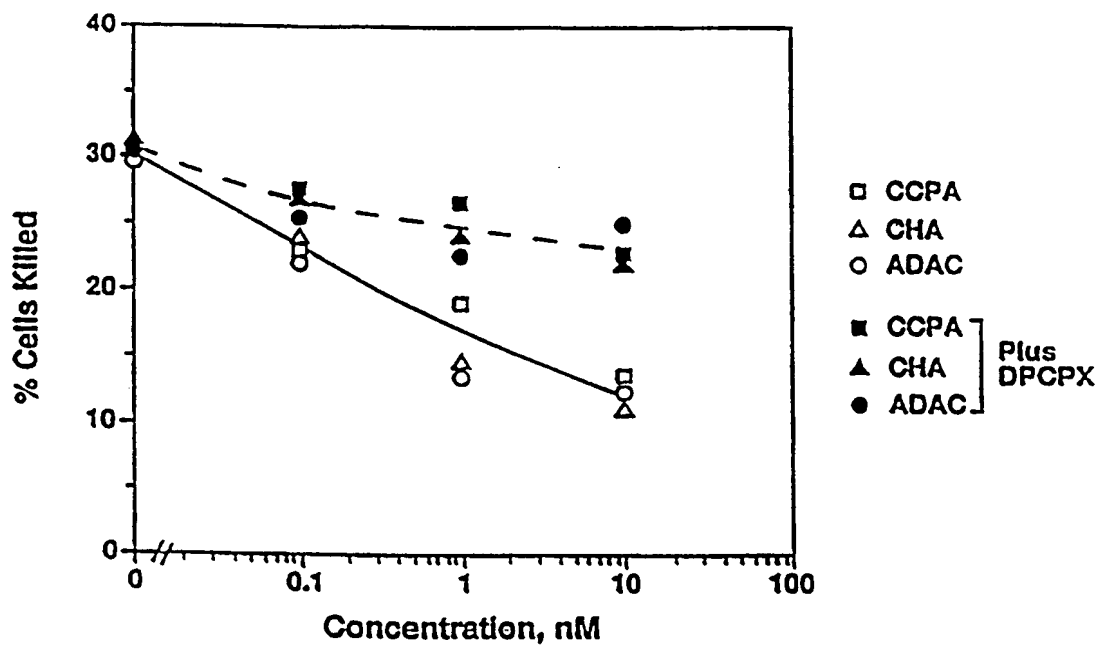
Figure 2

Fig. 3A
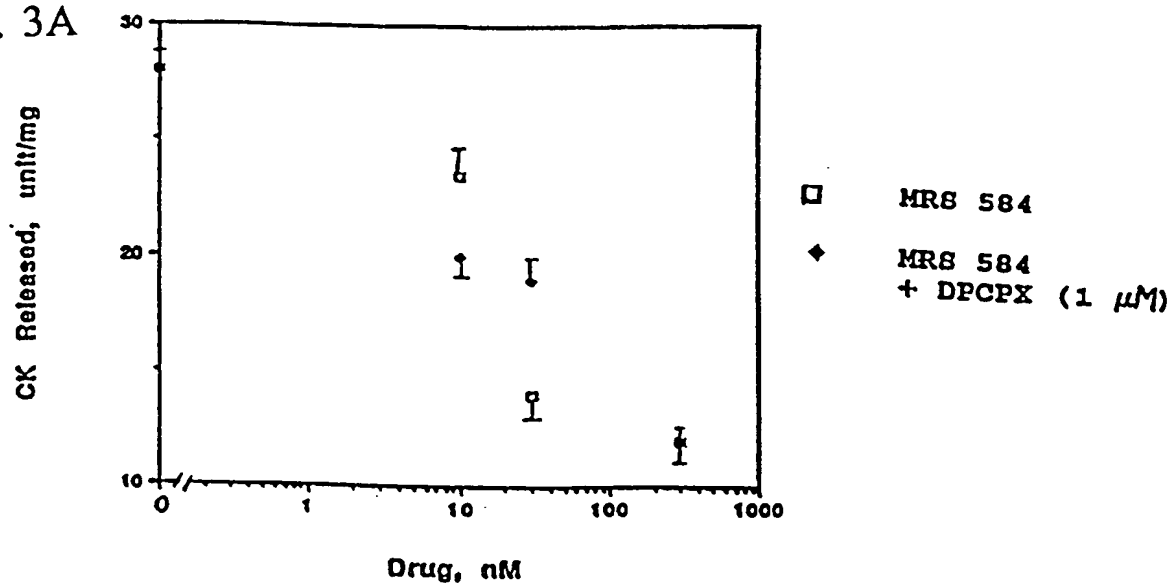
Fig. 3B
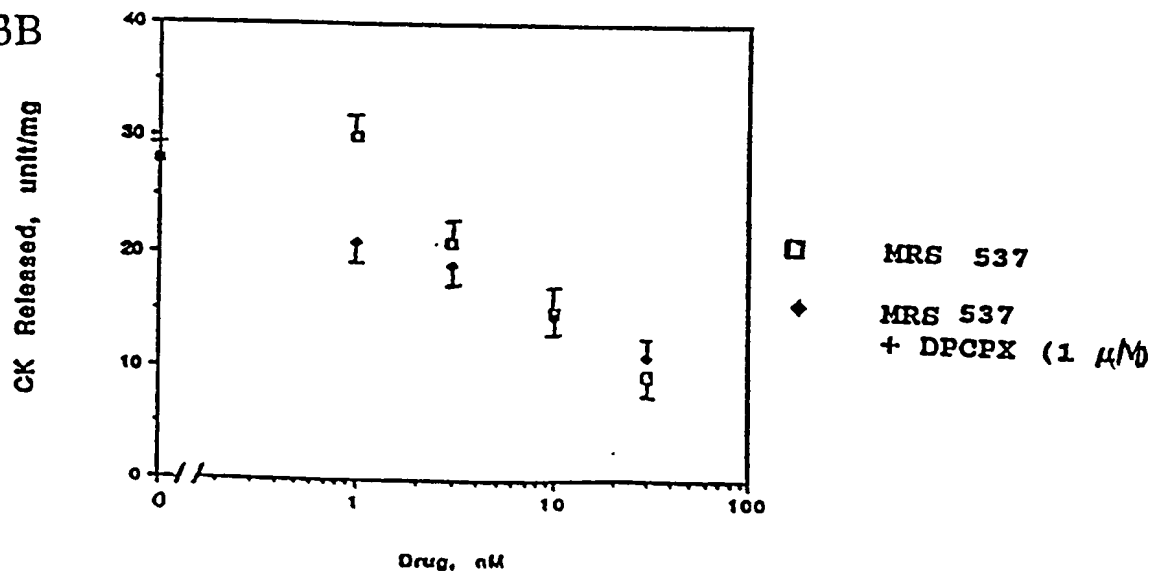
Fig. 3C
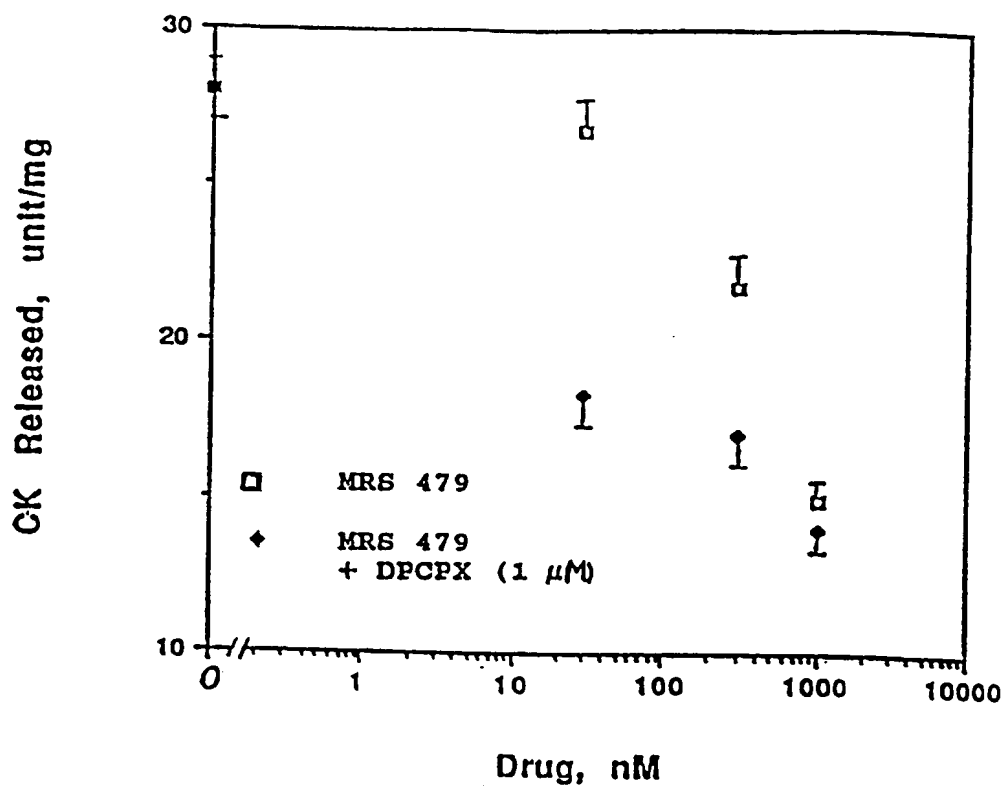
Fig. 3D
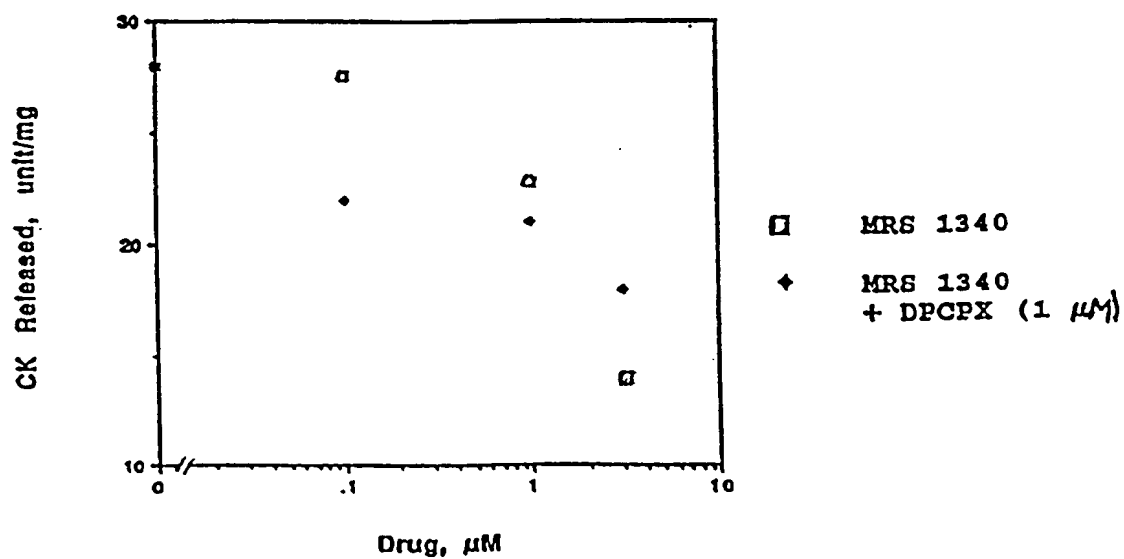
Fig. 3E

Fig. 3F

Fig. 4A

Fig. 4B
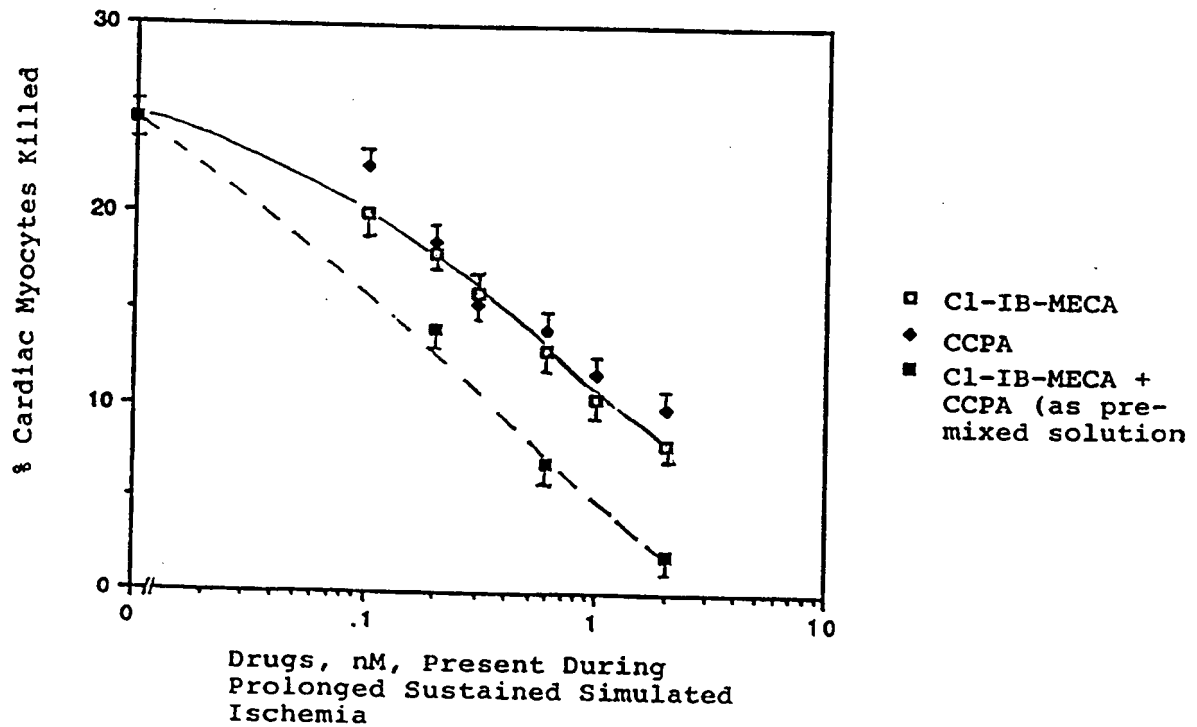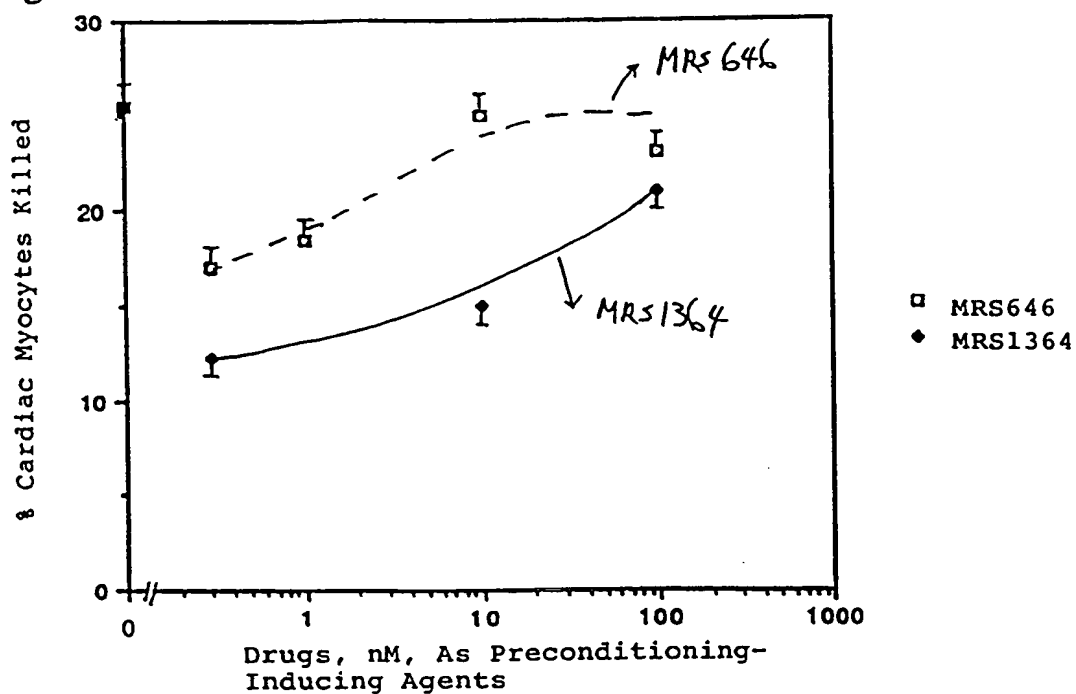
Fig. 5A

Fig. 5B

Fig. 5C

MRS1364, nM

Fig. 5D

MRS646, nM

Fig. 6A

MRS1543, nM

Fig. 6B

MRS1543, nM
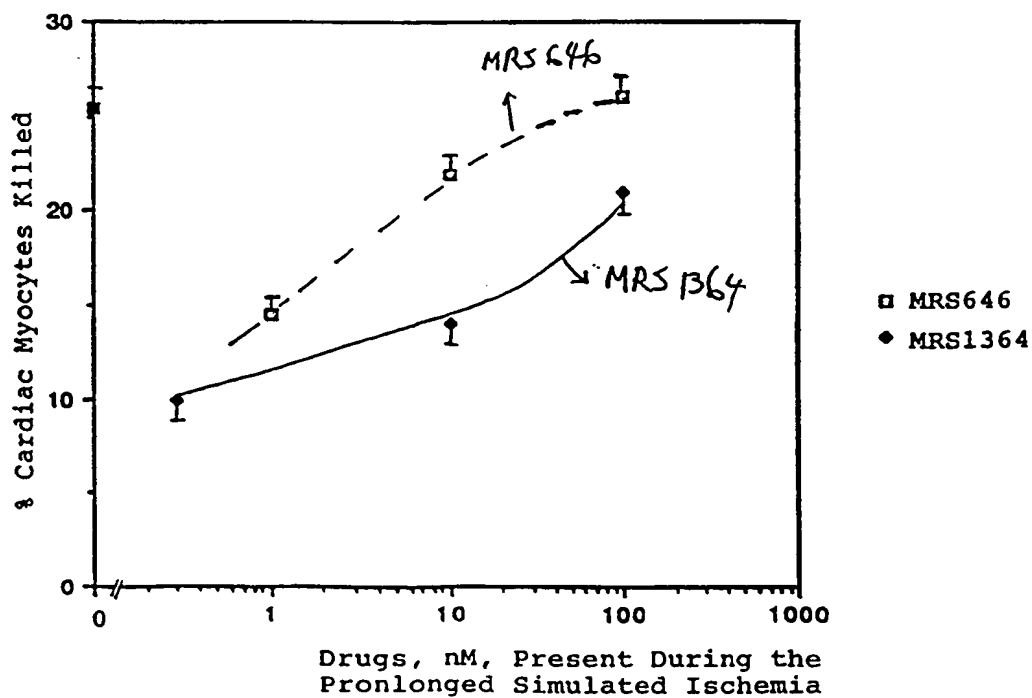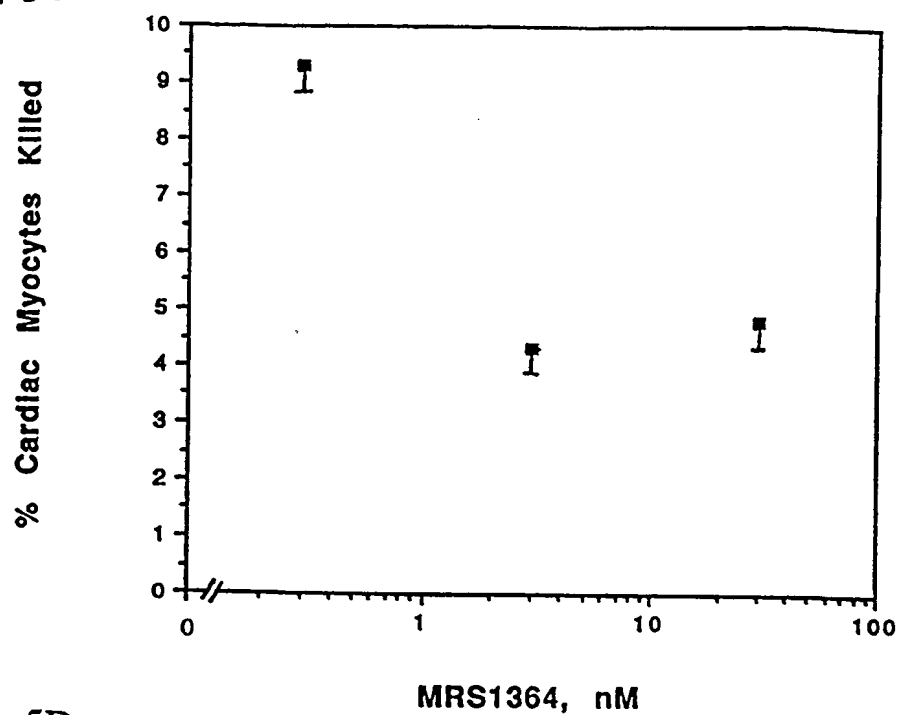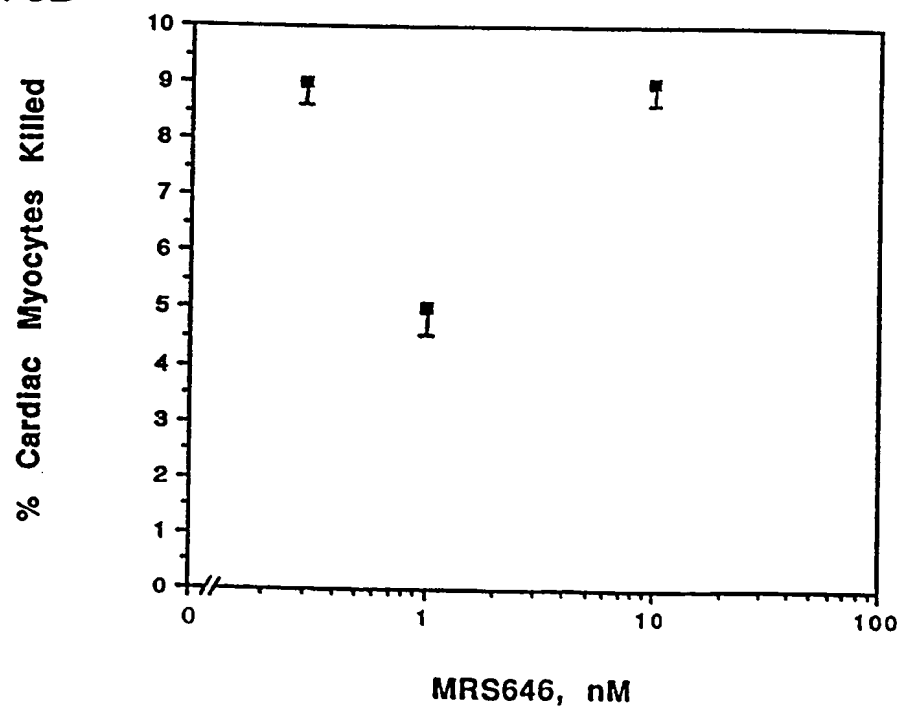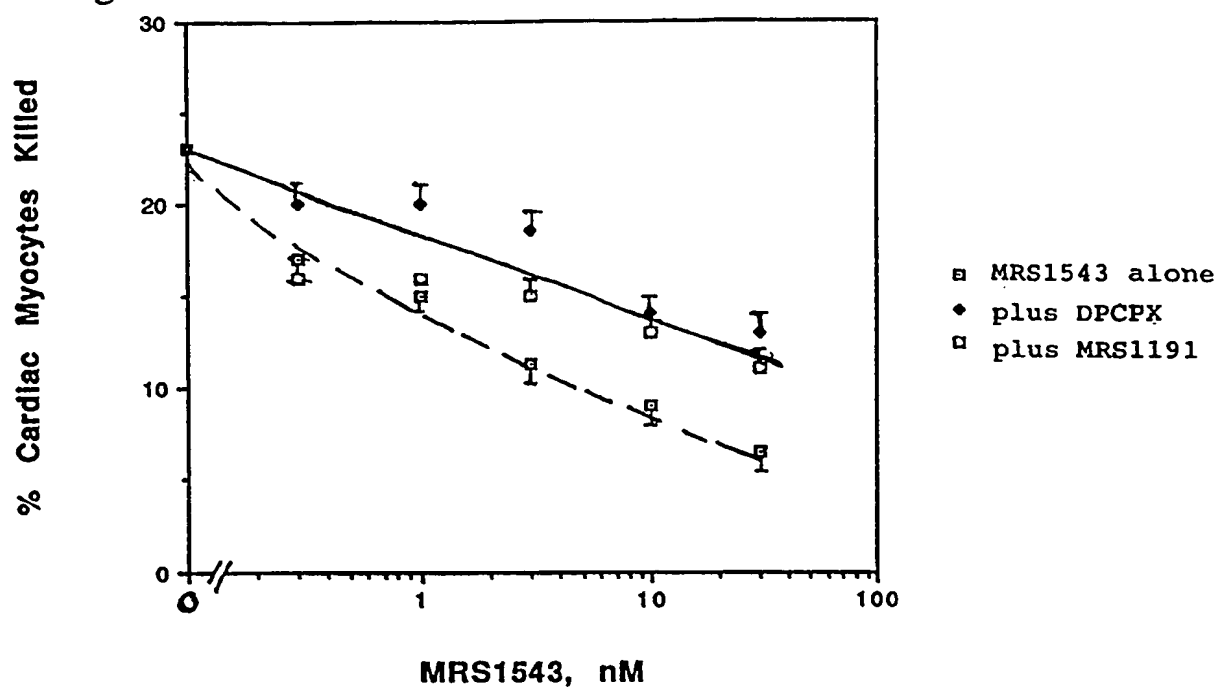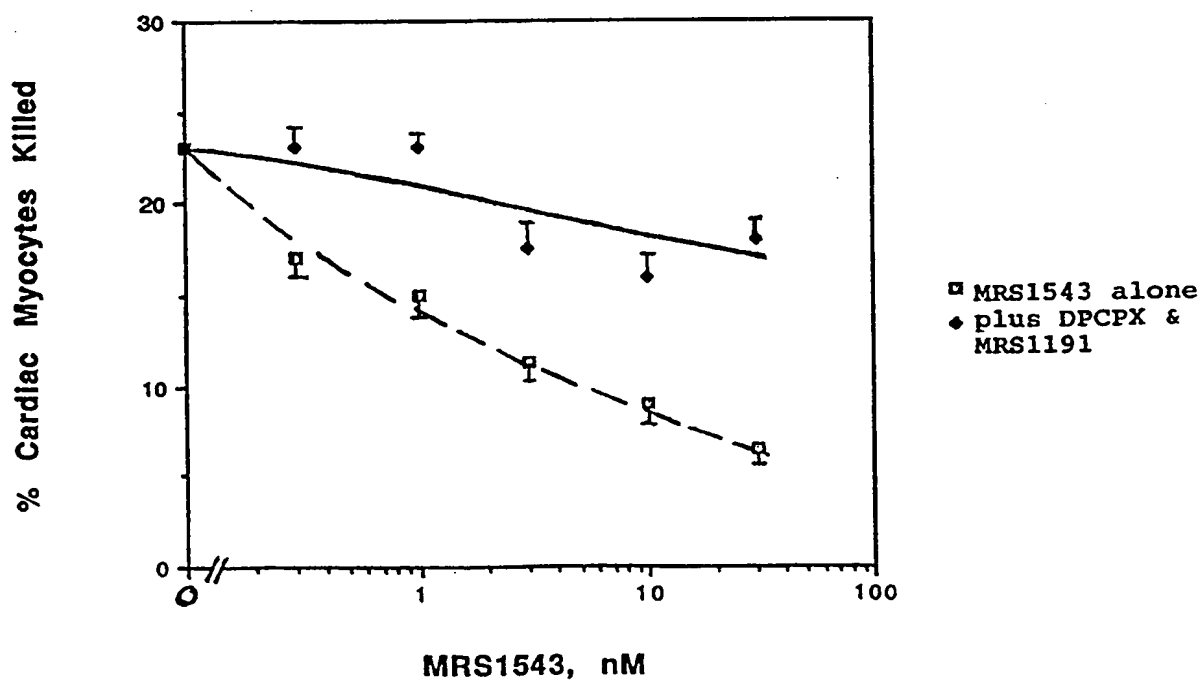
Fig. 6C
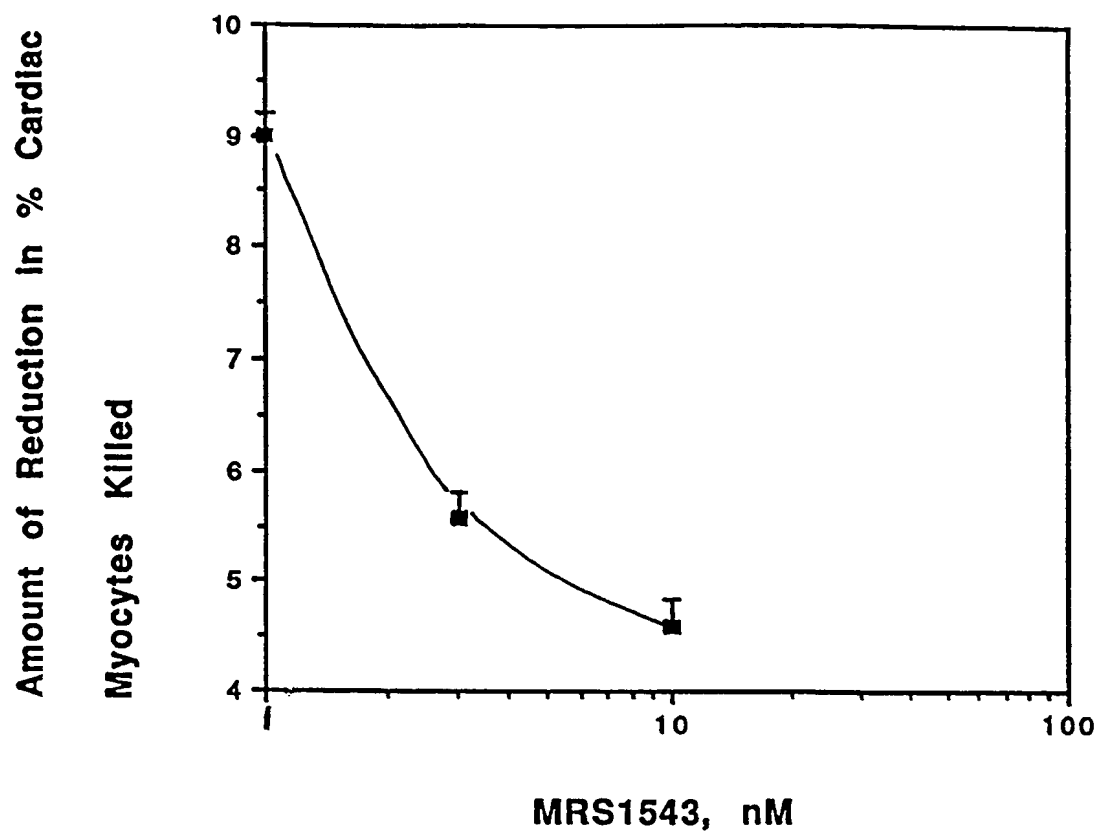
Fig. 7A
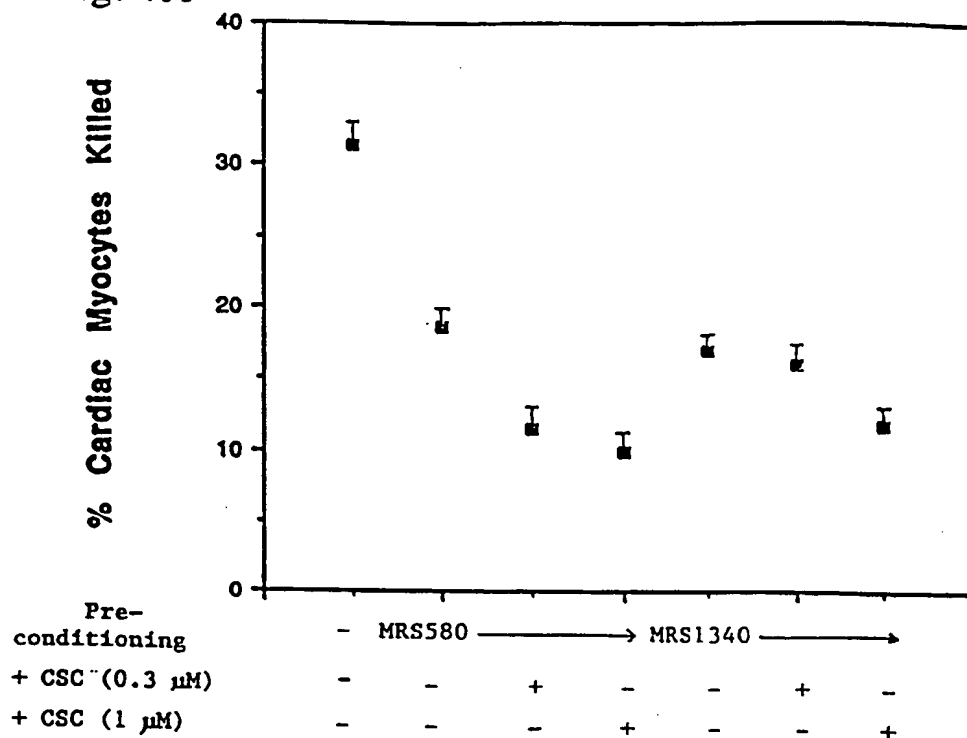
Fig. 7B
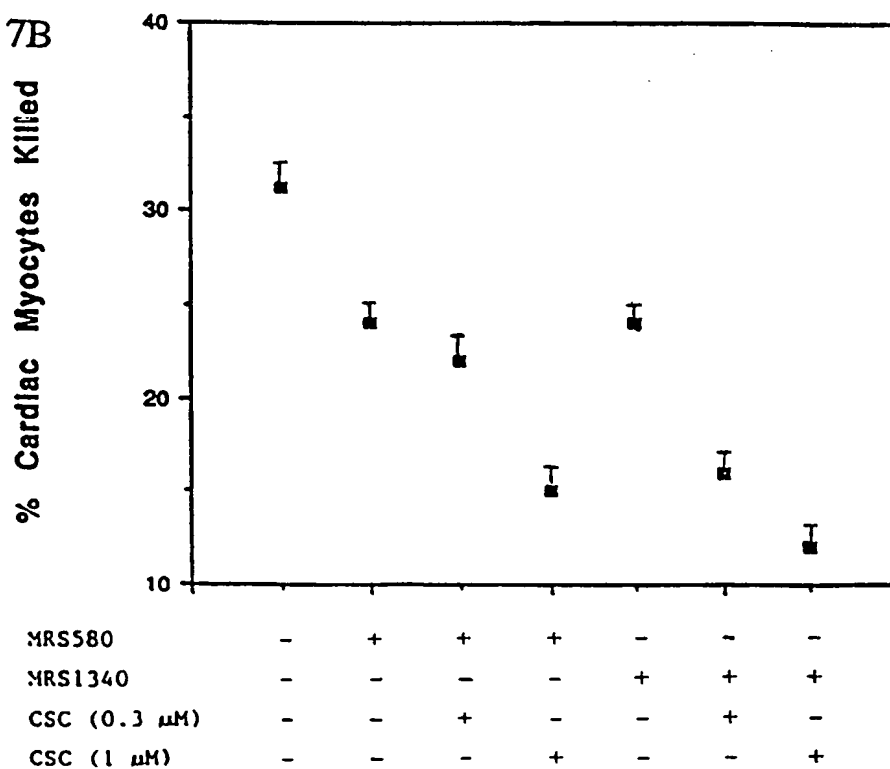
Fig. 7C
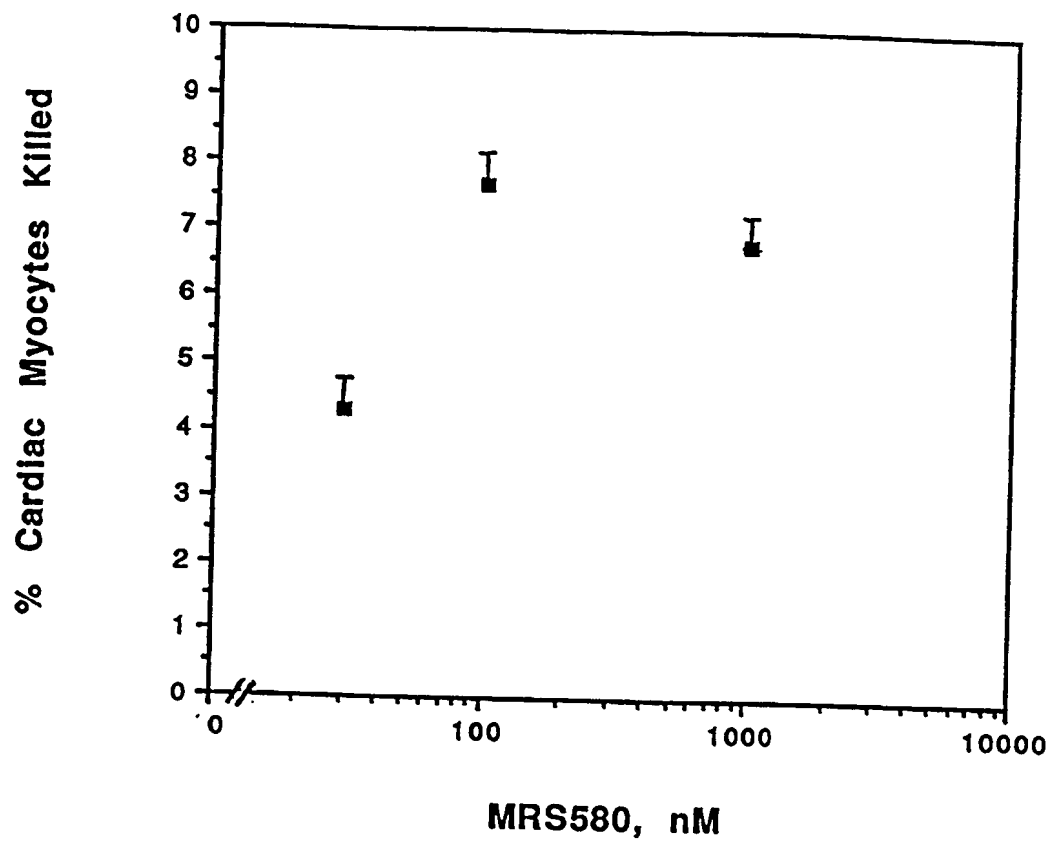
Fig. 8A
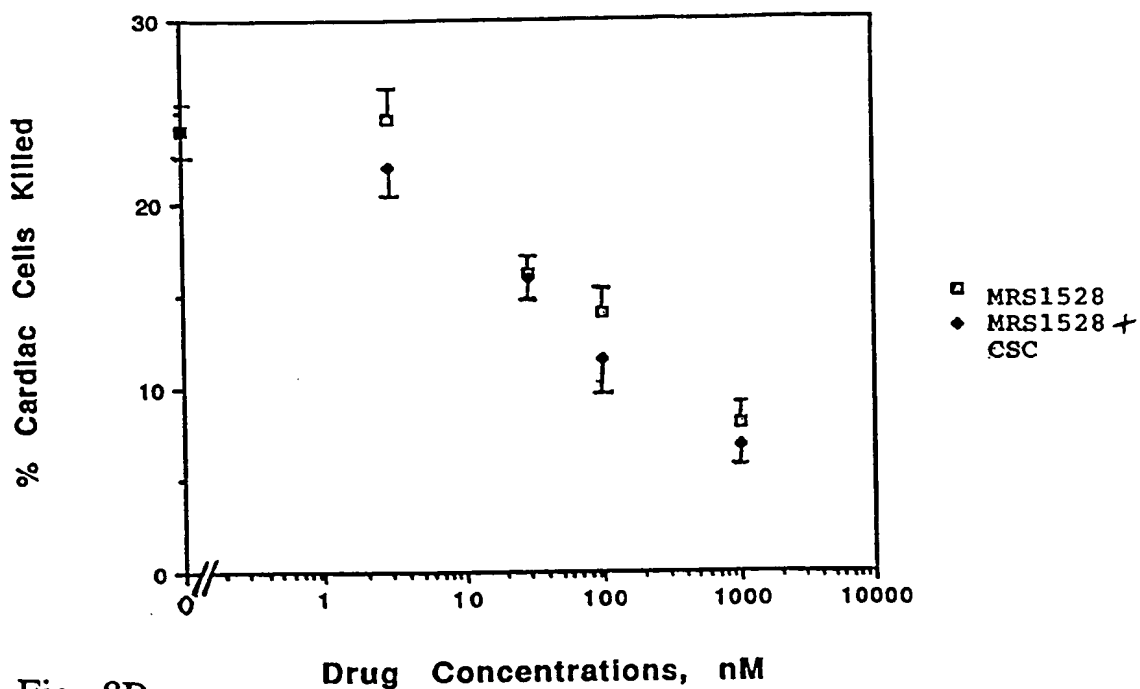
Fig. 8B
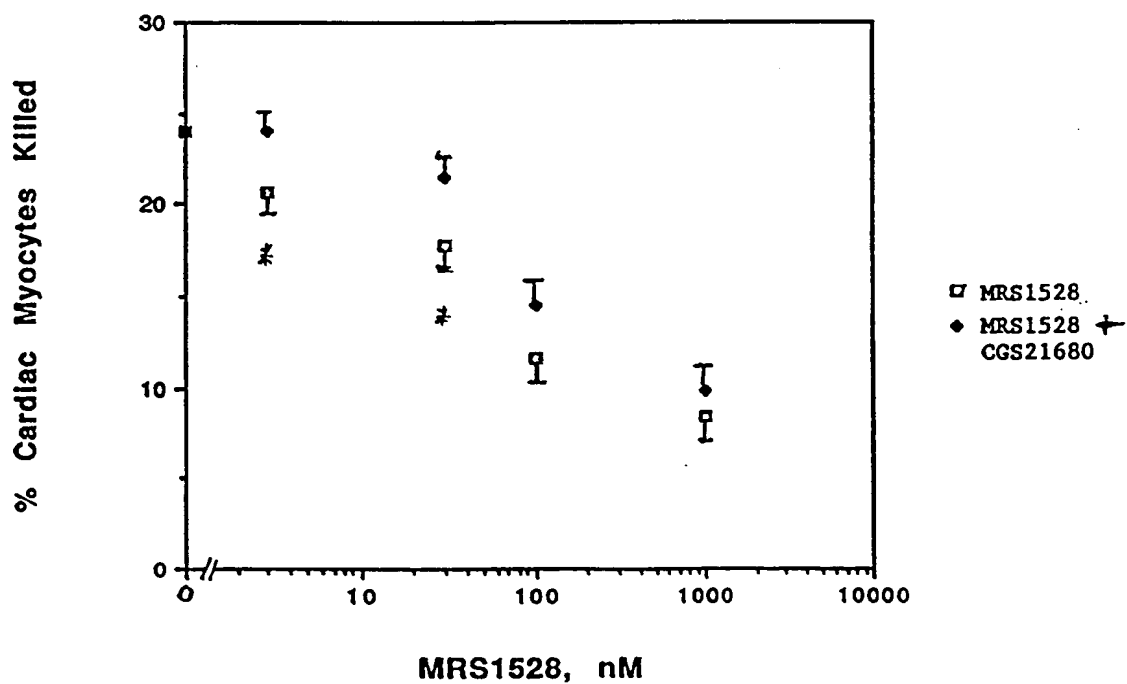
* significantly different from those determined in the presence of CGS21680

Fig. 8C

Drug Concentrations, nM

Fig. 8D

MRS1525, nM

Fig. 8E

R= H, IB-MECA

R= Cl, Cl-IB-MECA

III +

carbodiimide condensation

IV

V

III +

carbodiimide condensation

VI   n = 2-4

VII   n = 2-4

Figure 9

Fig. 10A **Derivatization of A₁ selective agonist for coupling to amine-derivatized A₃ agonist**

HN(CH₂)₂NHCOCH₂—⟨benzene⟩—NHCOCH₂—⟨benzene⟩—NH

NHCS

NCS

m- and p-DITC-ADAC

Derivatization of A₃ selective agonists

R'

NHCH₂—⟨benzene⟩

R—

CH₃NHCO

HO OH

R' = C—CCH₂NHR"; R = H, Cl

for coupling to isothiocaynates or carboxylic acids:

R" = H, COCH(R''')NH₂ [D- or L- amino acid],

for coupling to amines:

R" =CSNHC₆H₄-NCS (p- or m-),

COCH(R''')NHCSNHC₆H₄-NCS (p- or m-)

as A₃ agonists for exploring SAR:

R" =COCH₃, COCH(R''')NHCOOC(CH₃)₃ [Boc-D- or L- amino acid],

R''' = any naturally occurring amino acid

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Fig. 10B

Fig. 10C

Synthesis of binary conjugate with extended linker

linker region
(modulates affinity)

A₁-selective agonist pharmacophore

A_0-selective agonist pharmacophore
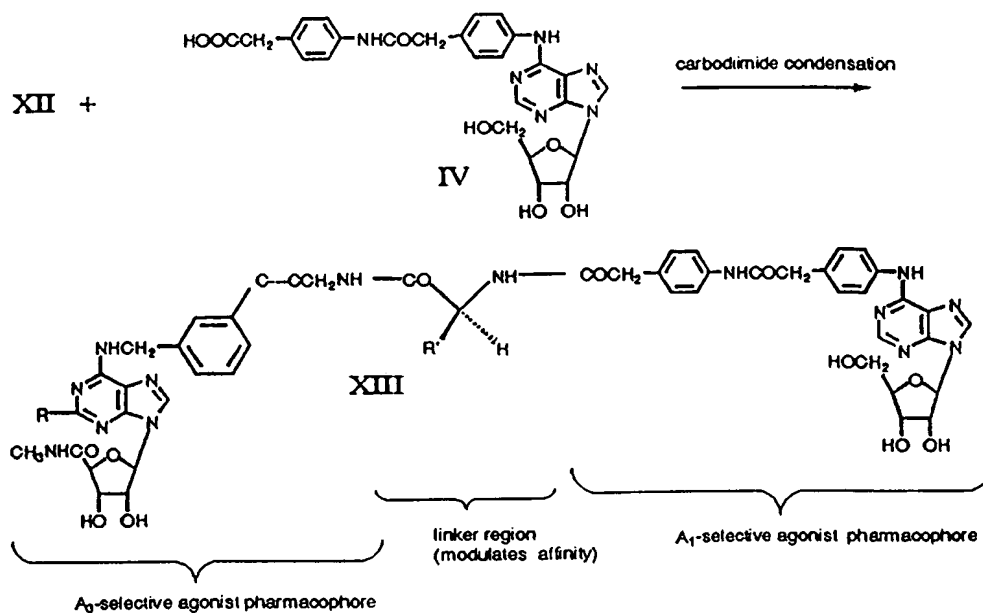
Synthesis of binary conjugate with short linker

linker region
(modulates affinity)

A_1-selective agonist pharmacophore

A_0-selective agonist pharmacophore
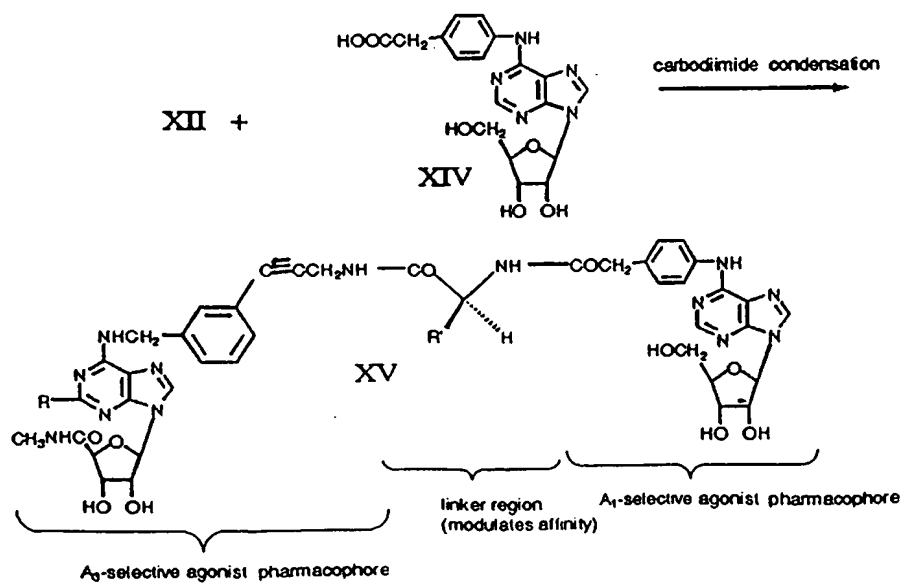
Fig. 11A

```
                    20                          40
AAG CTT GAT ATC GAA TTC CGC AGG ATG GTG CTT GCC TCG TGC CCC TTG

         60                    80
GTG CCC GTC TGC TGA TGT GCC CAG CCT GTG CCC GCC ATG CCG CCC TCC
                                                 M   P   P   S>

   100                   120                   140
ATC TCA GCT TTC CAG GCC GCC TAC ATC GGC ATC GAG GTG CTC ATC GCC
 I   S   A   F   Q   A   A   Y   I   G   I   E   V   L   I   A>

                  160                   180
CTG GTC TCT GTG CCC GGG AAC GTG CTG GTG ATC TGG GCG GTG AAG GTG
 L   V   S   V   P   G   N   V   L   V   I   W   A   V   K   V>

      200                   220                   240
AAC CAG GCG CTG CGG GAT GCC ACC TTC TGC TTC ATC GTC TCG CTG GCG
 N   Q   A   L   R   D   A   T   F   C   F   I   V   S   L   A>

                  260                   280
GTG GCT GAT GTG GCC GTG GGT GCC CTG GTC ATC CCC CTC GCC ATC CTC
 V   A   D   V   A   V   G   A   L   V   I   P   L   A   I   L>

      300                   320
ATC AAC ATT GGG CCA CAG ACC TAC TTC CAC ACC TGC CTC ATG GTT GCC
 I   N   I   G   P   Q   T   Y   F   H   T   C   L   M   V   A>

   340                   360                   380
TGT CCG GTC CTC ATC CTC ACC CAG AGC TCC ATC CTG GCC CTG CTG GCA
 C   P   V   L   I   L   T   Q   S   S   I   L   A   L   L   A>

              400                   420
ATT GCT GTG GAC CGC TAC CTC CGG GTC AAG ATC CCT CTC CGG TAC AAG
 I   A   V   D   R   Y   L   R   V   K   I   P   L   R   Y   K>

      440                   460                   480
ATG GTG GTG ACC CCC CGG AGG GCG GCG GTG GCC ATA GCC GGC TGC TGG
 M   V   V   T   P   R   R   A   A   V   A   I   A   G   C   W>

              500                   520
ATC CTC TCC TTC GTG GTG GGA CTG ACC CCT ATG TTT GGC TGG AAC AAT
 I   L   S   F   V   V   G   L   T   P   M   F   G   W   N   N>

      540                   560
CTG AGT GCG GTG GAG CGG GCC TGG GCA GCC AAC GGC AGC ATG GGG GAG
 L   S   A   V   E   R   A   W   A   A   N   G   S   M   G   E>

   580                   600                   620
CCC GTG ATC AAG TGC GAG TTC GAG AAG GTC ATC AGC ATG GAG TAC ATG
 P   V   I   K   C   E   F   E   K   V   I   S   M   E   Y   M>

              640                   660
```

Fig. 11B

```
GTC TAC TTC AAC TTC TTT GTG TGG GTG CTG CCC CCG CTT CTC CTC ATG
 V   Y   F   N   F   F   V   W   V   L   P   P   L   L   L   M>

      680                        700                        720
       *              *           *              *           *
GTC CTC ATC TAC CTG GAG GTC TTC TAC CTA ATC CGC AAG CAG CTC AAC
 V   L   I   Y   L   E   V   F   Y   L   I   R   K   Q   L   N>

              740                        760
               *                          *
AAG AAG GTG TCG GCC TCC TCC GGC GAC CCG CAG AAG TAC TAT GGG AAG
 K   K   V   S   A   S   S   G   D   P   Q   K   Y   Y   G   K>

              780                        800
     *         *              *           *
GAG CTG AAG ATC GCC AAG TCG CTG GCC CTC ATC CTC TTC CTC TTT GCC
 E   L   K   I   A   K   S   L   A   L   I   L   F   L   F   A>

   820                        840                        860
    *              *           *              *           *
CTC AGC TGG CTG CCT TTG CAC ATC CTC AAC TGC ATC ACC CTC TTC TGC
 L   S   W   L   P   L   H   I   L   N   C   I   T   L   F   C>

              880                        900
     *         *              *           *              *
CCG TCC TGC CAC AAG CCC AGC ATC CTT ACC TAC ATT GCC ATC TTC CTC
 P   S   C   H   K   P   S   I   L   T   Y   I   A   I   F   L>

   920                        940                        960
    *              *           *              *           *
ACG CAC GGC AAC TCG GCC ATG AAC CCC ATT GTC TAT GCC TTC CGC ATC
 T   H   G   N   S   A   M   N   P   I   V   Y   A   F   R   I>

              980                       1000
     *         *              *           *
CAG AAG TTC CGC GTC ACC TTC CTT AAG ATT TGG AAT GAC CAT TTC CGC
 Q   K   F   R   V   T   F   L   K   I   W   N   D   H   F   R>

        1020                      1040
    *        *                     *           *
TGC CAG CCT GCA CCT CCC ATT GAC GAG GAT CTC CCA GAA GAG AGG CCT
 C   Q   P   A   P   P   I   D   E   D   L   P   E   E   R   P>

   1060                      1080                      1100
    *              *           *           *              *
GAT GAC TAG ACC CCG CCT TCC GCT CCC ACC AGC CCA CAT CCA GTG GGG
 D   D   *>

              1120                      1140
               *              *           *
TCT CAG TCC AGT CCT CAC ATG CCC GCT GTC CCA GGG GTC TCC CTG AGC

   1160                      1180                      1200
    *              *              *           *           *
CTG CCC CAG CTG GGC TGT TGG CTG GGG GCA TGG GGG AGG CTC TGA AGA

              1220                      1240
     *         *              *           *
GAT ACC CAC AGA GTG TGG TCC CTC CAC TAG GAG TTA ACT ACC CTA CAC

        1260                      1280
    *        *              *           *              *
CTC TGG GCC CTG CAG GAG GCC TGG GAG GGA AGG GTC CTA CGG AGG GAC

   1300
    *
```

Fig. 12A

```
          10              20              30              40
           *               *               *               *
-CAA TTT TCA GCT GTT CTT TGC TCA ATA ATA ACT TTT TTA TCA CCA AGA

      50          60          70          80          90
       *           *           *           *           *
TAT CTC TCT AAG TTT TTG ACA TAT TCC TCA TTT GTT TTG ATA AAA GTT

      100         110         120         130         140
       *           *           *           *           *
TTC TTA TTT TCT TAG AAA AAT AAG TTA CTA AAG GTC ATA TAT CAT TGT

      150         160         170         180         190
       *           *           *           *           *
ATA TCT TCA AAA TAT TGC TTA AAA CTA GGA CTT GTA TTT AAA TGT TTT

      200         210         220         230         240
       *           *           *           *           *
TTC TTC TTA AAG ACA ATT TGC AGG TGC CCT CAG GAA CCC TGA AGC TGG

          250         260         270         280
           *           *           *           *
GCT GAG CCA TGA TGC TGC TGC CAG AAC CCC TGC AGA GGG CCT GGT TTC

  290         300         310         320         330
               *           *           *           *
AGG AGA CTC AGA GTC CTC TGT GAA AAA GCC CTT GGA GAG CGC CCC AGC

      340         350         360         370         380
       *           *           *           *           *
AGG GCT GCA CTT GGC TCC TGT GAG GAA GGG GCT CAG GGG TCT GGG CCC

      390         400         410         420         430
       *           *           *           *           *
CTC CGC CTG GGC CGG GCT GGG AGC CAG GCG GGC GGC TGG GCT GCA GCA

          440         450         460         470         480
               *           *           *           *           *
AAT GGA CCG TGA GCT GGC CCA GCC CGC GTC CGT GCT GAG CCT GCC TGT

          490         500         510         520         530
               *           *           *           *           *
CGT CTG TGG CC ATG CCC ATC ATG GGC TCC TCG GTG TAC ATC ACG GTG GAG
              M   P   I   M   G   S   S   V   Y   I   T   V   E>

          540         550         560         570
               *           *           *           *
CTG GCC ATT GCT GTG CTG GCC ATC CTG GGC AAT GTG CTG GTG TGC TGG
 L   A   I   A   V   L   A   I   L   G   N   V   L   V   C   W>

  580         590         600         610         620
   *           *           *           *           *
GCC GTG TGG CTC AAC AGC AAC CTG CAG AAC GTC ACC AAC TAC TTT GTG
 A   V   W   L   N   S   N   L   Q   N   V   T   N   Y   F   V>

      630         640         650         660         670
       *           *           *           *           *
GTG TCA CTG GCG GCG GCC GAC ATC GCA GTG GGT GTG CTC GCC ATC CCC
 V   S   L   A   A   A   D   I   A   V   G   V   L   A   I   P>

      680         690         700         710         720
       *           *           *           *           *
TTT GCC ATC ACC ATC AGC ACC GGG TTC TGC GCT GCC TGC CAC GGC TGC
```

Fig. 12B

```
          730              740              750              760              770
           *                *                *                *                *
CTC TTC ATT GCC TGC TTC GTC CTG GTC CTC ACG CAG AGC TCC ATC TTC
 L   F   I   A   C   F   V   L   V   L   T   Q   S   S   I   F>

               780              790              800              810
                *                *                *                *
AGT CTC CTG GCC ATC GCC ATT GAC CGC TAC ATT GCC ATC CGC ATC CCG
 S   L   L   A   I   A   I   D   R   Y   I   A   I   R   I   P>

  820              830              840              850              860
   *                *                *                *                *
CTC CGG TAC AAT GGC TTG GTG ACC GGC ACG AGG GCT AAG GGC ATC ATT
 L   R   Y   N   G   L   V   T   G   T   R   A   K   G   I   I>

     870              880              890              900              910
      *                *                *                *                *
GCC ATC TGC TGG GTG CTG TCG TTT GCC ATC GGC CTG ACT CCC ATG CTA
 A   I   C   W   V   L   S   F   A   I   G   L   T   P   M   L>

          920              930              940              950              960
           *                *                *                *                *
GGT TGG AAC AAC TGC GGT CAG CCA AAG GAG GGC AAG AAC CAC TCC CAG
 G   W   N   N   C   G   Q   P   K   E   G   K   N   H   S   Q>

               970              980              990              1000             1010
                *                *                *                *                *
GGC TGC GGG GAG GGC CAA GTG GCC TGT CTC TTT GAG GAT GTG GTC CCC
 G   C   G   E   G   Q   V   A   C   L   F   E   D   V   V   P>

               1020             1030             1040             1050
                *                *                *                *
ATG AAC TAC ATG GTG TAC TTC AAC TTC TTT GCC TGT GTG CTG GTG CCC
 M   N   Y   M   V   Y   F   N   F   F   A   C   V   L   V   P>

 1060             1070             1080             1090             1100
  *                *                *                *                *
CTG CTG CTC ATG CTG GGT GTC TAT TTG CGG ATC TTC CTG GCG GCG CGA
 L   L   L   M   L   G   V   Y   L   R   I   F   L   A   A   R>

    1110             1120             1130             1140             1150
     *                *                *                *                *
CGA CAG CTG AAG CAG ATG GAG AGC CAG CCT CTG CCG GGG GAG CGG GCA
 R   Q   L   K   Q   M   E   S   Q   P   L   P   G   E   R   A>

       1160             1170             1180             1190             1200
        *                *                *                *                *
CGG TCC ACA CTG CAG AAG GAG GTC CAT GCT GCC AAG TCA CTG GCC ATC
 R   S   T   L   Q   K   E   V   H   A   A   K   S   L   A   I>

          1210             1220             1230             1240             1250
           *                *                *                *                *
ATT GTT GGG CTC TTT GCC CTC TGC TGG CTG CCC CTA CAC ATC ATC AAC
 I   V   G   L   F   A   L   C   W   L   P   L   H   I   I   N>

               1260             1270             1280             1290
                *                *                *                *
TGC TTC ACT TTC TTC TGC CCC GAC TGC AGC CAC GCC CCT CTC TGG CTC
 C   F   T   F   F   C   P   D   C   S   H   A   P   L   W   L>

 1300             1310             1320             1330             1340
  *                *                *                *                *
ATG TAC CTG GCC ATC GTC CTC TCC CAC ACC AAT TCG GTT GTG AAT CCC
 M   Y   L   A   I   V   L   S   H   T   N   S   V   V   N   P>

     1350             1360             1370             1380             1390
      *                *                *                *                *
```

Fig. 12C

```
         TTC ATC TAC GCC TAC CGT ATC CGC GAG TTC CGC CAG ACC TTC CGC AAG
          F   I   Y   A   Y   R   I   R   E   F   R   Q   T   F   R   K>
             1400          1410          1420          1430          1440
              *             *             *             *             *
         ATC ATT CGC AGC CAC GTC CTG AGG CAG CAA GAA CCT TTC AAG GCA GCT
          I   I   R   S   H   V   L   R   Q   Q   E   P   F   K   A   A>
             1450          1460          1470          1480          1490
              *             *             *             *             *
         GGC ACC AGT GCC CGG GTC TTG GCA GCT CAT GGC AGT GTC GGA GAG CAG
          G   T   S   A   R   V   L   A   A   H   G   S   V   G   E   Q>
             1500          1510          1520          1530
              *             *             *             *
         GTC AGC CTC CGT CTC AAC GGC CAC CCG CCA GAG GTG TGG GCC AAC GGC
          V   S   L   R   L   N   G   H   P   P   E   V   W   A   N   G>
        1540          1550          1560          1570          1580
         *             *             *             *             *
         AGT GCT CCC CAC CCT GAG CGG AGG CCC AAT GGC TAC GCC CTG GGG CTG
          S   A   P   H   P   E   R   R   P   N   G   Y   A   L   G   L>
             1590          1600          1610          1620          1630
              *             *             *             *             *
         GTG AGT GGA GGG AGT GCC CAA GAG TCC CAG GGG AAC ACG GGC CTC CCA
          V   S   G   G   S   A   Q   E   S   Q   G   N   T   G   L   P>
             1640          1650          1660          1670          1680
              *             *             *             *             *
         GAC GTG GAG CTC CTT AGC CAT GAG CTC AAG AGA GTG TGC CCA GAG CCC
          D   V   E   L   L   S   H   E   L   K   R   V   C   P   E   P>
             1690          1700          1710          1720          1730
              *             *             *             *             *
         CCT GGC CTA GAT GAC CCC CTG GCC CAG GAT GGA GCA GGA GTG TCC TGA T
          P   G   L   D   D   P   L   A   Q   D   G   A   G   V   S   *>
             1740          1750          1760          1770
              *             *             *             *
         GAT TCA TGG AGT TTG CCC CTT CCT AAG GGA AGG AGA TCT TTA TCT TTC
        1780          1790          1800          1810          1820
         *             *             *             *             *
         TGG TTG GCT TGA CCA GTC ACG TTG GGA GAA GAG AGA GAG TGC CAG GAG
        1830          1840          1850          1860          1870
         *             *             *             *             *
         ACC CTG AGG GCA GCC GGT TCC TAC TTT GGA CTG AGA GAA GGG AGC CCC
             1880          1890          1900          1910          1920
              *             *             *             *             *
         AGG CTG GAG CAG CAT GAG GCC CAG CAA GAA GGG CTT GGG TTC TGA GGA
             1930          1940          1950          1960          1970
              *             *             *             *             *
         AGC AGA TGT TTC ATG CTG TGA GGC CTT GCA CCA GGT GGG GGC CAC AGC
             1980          1990          2000          2010
              *             *             *             *
         ACC AGC AGC ATC TTT GCT GGG CAG GGC CCA GCC CTC CAC TGC AGA AGC
        2020          2030          2040          2050          2060
         *             *             *             *             *
         ATC TGG AAG CAC CAC CTT GTC TCC ACA GAG CAG CTT GGG CAC AGC AGA
        2070          2080          2090          2100          2110
```

Fig. 12 D

CTG GCC TGG CCC TGA GAC TGG GGA GTG GCT CCA ACA GCC TCC TGC CAC

2120   2130   2140   2150   2160

CCA CAC ACC ACT CTC CCT AGA CTC TCC TAG GGT TCA GGA GCT GCT GGG

2170   2180   2190   2200   2210

CCC AGA GGT GAC ATT TGA CTT TTT TTC CAG GAA AAA TGT AAG TGT GAG

2220   2230   2240   2250

GAA ACC CTT TTT ATT TTA TTA CCT TTC ACT CTC TGG CTG CTG GGT CTG

2260   2270   2280   2290   2300

CCG TCG GTC CTG CTG CTA ACC TGG CAC CAG AGC CTC TGC CCG GGG AGC

2310   2320   2330   2340   2350

CTC AGG CAG TCC TCT CCT GCT GTC ACA GCT GCC ATC CAC TTC TCA GTC

2360   2370   2380   2390   2400

CCA GGG CCA TCT CTT GGA GTG ACA AAG CTG GGA TCA AGG ACA GGG AGT

2410   2420   2430   2440   2450

TGT AAC AGA GCA GTG CCA GAG CAT GGG CCC AGG TCC CAG GGG AGA GGT

2460   2470   2480   2490

TGG GGC TGG CAG GCC ACT GGC ATG TGC TGA GTA GCG CAG AGC TAC CCA

2500   2510   2520   2530   2540

GTG AGA GGC CTT GTC TAA CTG CCT TTC CTT CTA AAG GGA ATG TTT TTT

2550   2560   2570

TCT GAG ATA AAA TAA AAA CGA GCC ACA G

Fig. 13A

```
        10          20          30          40          50          60
         *           *           *           *           *           *
-GGACCTCTGGGAAGACGTCTGGCGAGAGCTAGGCCCACTGGCCCTACAGACGGATCTTGC

        70          80          90         100         110
         *           *           *           *           *
TGGCTCACCTGTCCCTGTGGAGGTTCCCCTGGGAAGGCAAG ATG CCC AAC AAC
                                            Met Pro Asn Asn>

        120         130         140         150
         *           *           *           *
AGC ACT GCT CTG TCA TTG GCC AAT GTT ACC TAC ATC ACC ATG GAA
Ser Thr Ala Leu Ser Leu Ala Asn Val Thr Tyr Ile Thr Met Glu>

160         170         180         190         200
 *           *           *           *           *
ATT TTC ATT GGA CTC TGC GCC ATA GTG GGC AAC GTG CTG GTC ATC
Ile Phe Ile Gly Leu Cys Ala Ile Val Gly Asn Val Leu Val Ile>

        210         220         230         240
         *           *           *           *
TGC GTG GTC AAG CTG AAC CCC AGC CTG CAG ACC ACC ACC TTC TAT
Cys Val Val Lys Leu Asn Pro Ser Leu Gln Thr Thr Thr Phe Tyr>

250         260         270         280         290
 *           *           *           *           *
TTC ATT GTC TCT CTA GCC CTG GCT GAC ATT GCT GTT GGG GTG CTG
Phe Ile Val Ser Leu Ala Leu Ala Asp Ile Ala Val Gly Val Leu>

        300         310         320         330
         *           *           *           *
GTC ATG CCT TTG GCC ATT GTT GTC AGC CTG GGC ATC ACA ATC CAC
Val Met Pro Leu Ala Ile Val Val Ser Leu Gly Ile Thr Ile His>

340         350         360         370         380
 *           *           *           *           *
TTC TAC AGC TGC CTT TTT ATG ACT TGC CTA CTG CTT ATC TTT ACC
Phe Tyr Ser Cys Leu Phe Met Thr Cys Leu Leu Leu Ile Phe Thr>

        390         400         410         420
         *           *           *           *
CAC GCC TCC ATC ATG TCC TTG CTG GCC ATC GCT GTG GAC CGA TAC
His Ala Ser Ile Met Ser Leu Leu Ala Ile Ala Val Asp Arg Tyr>

430         440         450         460         470
 *           *           *           *           *
TTG CGG GTC AAG CTT ACC GTC AGA TAC AAG AGG GTC ACC ACT CAC
Leu Arg Val Lys Leu Thr Val Arg Tyr Lys Arg Val Thr Thr His>

        480         490         500         510
         *           *           *           *
AGA AGA ATA TGG CTG GCC CTG GGC CTT TGC TGG CTG GTG TCA TTC
Arg Arg Ile Trp Leu Ala Leu Gly Leu Cys Trp Leu Val Ser Phe>

520         530         540         550         560
 *           *           *           *           *
CTG GTG GGA TTG ACC CCC ATG TTT GGC TGG AAC ATG AAA CTG ACC
Leu Val Gly Leu Thr Pro Met Phe Gly Trp Asn Met Lys Leu Thr>

        570         580         590         600
```

Fig. 13B

```
                *                  *                    *                    *
        TCA GAG TAC CAC AGA AAT GTC ACC TTC CTT TCA TGC CAA TTT GTT
        Ser Glu Tyr His Arg Asn Val Thr Phe Leu Ser Cys Gln Phe Val>

610             620             630             640             650
  *               *               *               *               *
        TCC GTC ATG AGA ATG GAC TAC ATG GTA TAC TTC AGC TTC CTC ACC
        Ser Val Met Arg Met Asp Tyr Met Val Tyr Phe Ser Phe Leu Thr>

                660             670             680             690
                  *               *               *               *
        TGG ATT TTC ATC CCC CTG GTT GTC ATG TGC GCC ATC TAT CTT GAC
        Trp Ile Phe Ile Pro Leu Val Val Met Cys Ala Ile Tyr Leu Asp>

700             710             720             730             740
  *               *               *               *               *
        ATC TTT TAC ATC ATT CGG AAC AAA CTC AGT CTG AAC TTA TCT AAC
        Ile Phe Tyr Ile Ile Arg Asn Lys Leu Ser Leu Asn Leu Ser Asn>

                750             760             770             780
                  *               *               *               *
        TCC AAA GAG ACA GGT GCA TTT TAT GGA CGG GAG TTC AAG ACG GCT
        Ser Lys Glu Thr Gly Ala Phe Tyr Gly Arg Glu Phe Lys Thr Ala>

790             800             810             820             830
  *               *               *               *               *
        AAG TCC TTG TTT CTG GTT CTT TTC TTG TTT GCT CTG TCA TGG CTG
        Lys Ser Leu Phe Leu Val Leu Phe Leu Phe Ala Leu Ser Trp Leu>

                840             850             860             870
                  *               *               *               *
        CCT TTA TCT ATC ATC AAC TGC ATC ATC TAC TTT AAT GGT GAG GTA
        Pro Leu Ser Ile Ile Asn Cys Ile Ile Tyr Phe Asn Gly Glu Val>

880             890             900             910             920
  *               *               *               *               *
        CCA CAG CTT GTG CTG TAC ATG GGC ATC CTG CTG TCC CAT GCC AAC
        Pro Gln Leu Val Leu Tyr Met Gly Ile Leu Leu Ser His Ala Asn>

                930             940             950             960
                  *               *               *               *
        TCC ATG ATG AAC CCT ATC GTC TAT GCC TAT AAA ATA AAG AAG TTC
        Ser Met Met Asn Pro Ile Val Tyr Ala Tyr Lys Ile Lys Lys Phe>

970             980             990             1000            1010
  *               *               *               *               *
        AAG GAA ACC TAC CTT TTG ATC CTC AAA GCC TGT GTG GTC TGC CAT
        Lys Glu Thr Tyr Leu Leu Ile Leu Lys Ala Cys Val Val Cys His>

                1020            1030            1040            1050
                  *               *               *               *
        CCC TCT GAT TCT TTG GAC ACA AGC ATT GAG AAG AAT TCT GAG TAG
        Pro Ser Asp Ser Leu Asp Thr Ser Ile Glu Lys Asn Ser Glu ***>

1060        1070        1080        1090        1100        1110
  *           *           *           *           *           *
        TTATCCATCAGAGATGACTCTGTCTCATTGACCTTCAGATTCCCCATCAACAAACACTTG

1120        1130        1140        1150        1160        1170
  *           *           *           *           *           *
        AGGGCCTGTATGCCTGGGCCAAGGGATTTTTTACATCCTTGATTACTTCCACTGAGGTGGG
```

Fig. 13 C

```
       1180        1190        1200        1210        1220        1230
        *           *           *           *           *           *
      AGCATCTCCAGTGCTCCCCAATTATATCTCCCCCACTCCACTACTCTCTTCCTCCACTTC

       1240        1250        1260        1270        1280        1290
        *           *           *           *           *           *
      ATTTTTCCTTTGTCCTTTCTCTCTAATTCAGTGTTTTGGAGGCCTGACTTGGGGACAACG

       1300       .1310        1320        1330        1340        1350
        *           *           *           *           *           *
      TATTATTGATATTATTGTCTGTTTTCCTTCTTCCCAATAGAAGAATAAGTCATGGAGCCT

       1360        1370
        *           *
      GAAGGGTGCCTAGTTGAC
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09031

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :A61K 31/70
US CL   : 514/46

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   514/46

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

   NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

   CAS ONLINE, DERWENT

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,657,897 A (BRISTOL et al.) 14 April 1987, see entire document.	1-63

☐   Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	
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Date of the actual completion of the international search	Date of mailing of the international search report
16 SEPTEMBER 1998	20 OCT 1998

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